Formulation of DNA condensed by lipopolyamines

Ahmed, Osama A. A.

Award date:
2006

Awarding institution:
University of Bath

Formulation of DNA condensed by lipopolyamines

Osama A. A. Ahmed

A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Pharmacy and Pharmacology

June 2006

Signed:.....

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Abstract

In this work we investigate the design and formulation of novel non-viral lipopolyamine vectors capable of efficiently and safely delivering DNA to the nucleus in different tissue cultured cell lines. The thesis starts with a literature review about NVGT that provides a background about the barriers for DNA delivery and a focus on the NVGT vectors used in the formulation of DNA.

The first lipopolyamine in this study is N^4,N^9-dioleoyl spermine that was synthesized from the naturally occurring polyamine spermine. To investigate the ability of N^4,N^9-dioleoyl spermine on DNA condensation using ethidium bromide (EthBr) fluorescence-quenching and light scattering assays, model polycations were selected to be compared with our lipopolyamine. Transfection efficiency and cytotoxicity (using MTT) were studied in both cancer and primary cell lines and compared with Lipofectin® and Lipofectamine™. N^4,N^9-Dioleoyl spermine formula is efficient at condensing and transfecting DNA at a charge ratios of 2.5.

We then extended our study to the effect of varying the degree of unsaturation in N^4,N^9-dioctadecanoyl spermines as non-viral vectors. The saturated (stearoyl), mono-unsaturated (oleoyl) and the di-unsaturated (linoleoyl) were synthesized. The ability of these compounds to condense DNA was studied using EthBr and nanoparticle characterization techniques. Transfection efficiency was studied in a panel of primary skin cells (FEK4, FCP4, FCP5, FCP7, and FCP8) and in cancer cell line (HtTA), and compared with the lipospermine Transfectam®. N^4,N^9-Dilinoleoyl spermine also achieves the highest transfection levels among the studied lipopolyamines in a series of primary skin cells and cancer cell lines at low charge ratios of (+/- ammonium/phosphate) 5.5.

Two refereed papers, one edited book chapter and several abstracts of this study presented at international meetings are included in the appendix.

Acknowledgements

I would like to thank Dr. Ian S. Blagbrough for his continuous guidance and support without which this work would not have been possible.

I would also like to thank Dr. Charareh Pourzand for her advice and support in biological evaluation studies.

I am grateful to Prof. R. M. Tyrrell (University of Bath) for FEK4 and HtTA cell lines.

I also thank Dr. J.M.H. van den Elsen (University of Bath) for generous help with the particle-size measurements (funded under a BBSRC grant BBS/B/12121).

I thank the research and technical staff at Department of Pharmacy and Pharmacology: Kevin Smith, Jo Carter, Don Perry, Patricia Holly, Dr. Anthie Yiakouvaki and Dr. May Al-Nabaheen.

I also gratefully acknowledge the financial support of this work from the Egyptian Government.

Finally, I would like to thank my family and my colleagues in the Department for their continuing support.

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Abbreviations

bp	Base pair
CMV	Cyto Megalo Virus
DLenDMA	1,2-Dilinolenyloxy-*N*,*N*-dimethyl-3-aminopropane
DLinDMA	1,2-Dilinoleyloxy-*N*,*N*-dimethyl-3-aminopropane
DCC	Dicyclohexylcarbodiimide
DDAB	Didodecyl dimethylammonium bromide
DCU	Dicyclohexylurea
DMSO	Dimethylsulfoxide
DODMA	1,2-Dioleyloxy-*N*,*N*-dimethyl-3-aminopropane
DOGS	Dioctadecylamidoglycylspermine
DOPC	Dioleoylphosphatidyl choline
DOPE	Dioleoylphosphatidyl ethanolamine
DOSPA	2,3-Dioleyloxy-*N*-[2(spermine-carboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate
DOSPER	1,3-Dioleoyloxy-2-(6-carboxyspermine)
DOTMA	2,3-Dioleyloxypropyl-1-trimethyl ammonium chloride
DSDMA	1,2-Distearyloxy-*N*,*N*-dimethyl-3-aminopropane
EDTA	Ethylenediamine tetra-acetic acid
EGFP	Enhanced green fluorescent protein
E. coli	*Escherichia coli*
EMEM	Earle's minimal essential medium
EthBr	Ethidium bromide
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
HEPES	*N*-(2-Hydroxyethyl)piperazine-*N*'-2-ethansulfonic acid
HOBt	Hydroxybenzotriazole
HRMS	High-resolution mass spectroscopy
LB	Luria Bertani
MeOH	Methanol

MOPS	3-[*N*-Morpholino] propanesulfonic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NLP	Nano-lipoparticle
NLS	Nuclear localization signal
NMR	Nuclear magnetic resonance
N/P	Ammonium/phosphate
NPC	Nuclear pore complex
NVGT	Non-viral gene therapy
PBS	Phosphate Buffered Saline
PE	Phosphatidyl ethanolamine
PEG	Polyethyleneglycol
PEI	Polyethylenimine
PLL	Poly-L-lysine
PS	Phosphatidylserine
SV	Simian Virus
TAE	Tris-Acetate-EDTA
TLC	Thin layer chromatography
TREN	Tris (2-aminoethyl) amine
UV	Ultra-violet

CHAPTER 1

Introduction and Literature Review

Introduction

It is widely believed that gene therapy will become an efficient medicine for the treatment of diseases such as cancer, cystic fibrosis and for vaccination (1). For example in the case of cancer, the cells are characterized by their uncontrolled growth through their characteristic hereditary properties (2). The current strategies (surgery, chemotherapy and/or radiation) for cancer treatment are useful in the case of early stages of cancer, but in the late metastases stage although chemotherapy is the most successful strategy, it has several side-effects e.g. the suppression of bone marrow and other fast dividing tissues, potential genesis of secondary cancers, and the development of resistant phenotypes (3).

The essential requirements for gene therapy are the transport of DNA through the cell membrane and ultimately to the nucleus. Different strategies have been used for the delivery of genetic material into target cells which are classified as viral or non-viral delivery systems (4, 5). Viral delivery systems depend on the development of genetically-modified viruses to utilise their capability of efficiently delivering DNA into cells through their natural infective mechanisms without their pathogenic characteristics. Although high efficiency is achieved by viral vectors, there are concerns about their use which include a limit to the size of the DNA to be delivered (the "payload"), endogenous viral recombination, unexpected anti-vector immune response, and oncogene activation (6-8). Viral gene therapy has suffered a major blow by the first fatal case, the death of 18-year-old Jesse Gelsinger in a clinical trial after starting the treatment using adenoviral vector in 1999 (9). Also, in 2003 a child treated in a French viral gene therapy trial developed a leukemia-like condition that made the FDA temporarily halt all gene therapy trials using retroviral vectors (9). These set-backs for viral vectors have served to attract several laboratories to focus on the development of novel non-viral formulations for efficient gene delivery.

Non-viral delivery systems include the use of DNA alone, so-called "naked DNA", or complexed with synthetic cationic lipids (lipoplexes) or cationic polymers (polyplexes) (5). These vectors not only circumvent the drawbacks of viral vectors, but also have the advantages of simplicity of use and the ease of large-scale production (10). The major challenge for non-viral vectors is the lower transfection

efficiency compared to viral vectors. In terms of concept, the naked DNA strategy is considered as the safest way (10), but due to the limitations of level of expression, other procedures were used to increase the transfection efficiency such as electroporation (11), gene gun (12), ultrasound (13) and hydrodynamic (high pressure) injection (14), but these techniques also suffer from the need for complicated medical devices for application and the limitation of only achieving drug (gene) delivery to the superficial tissues.

Synthetic non-viral gene carriers (lipoplexes and polyplexes) are widely investigated due to their advantages which include the variety of ways for synthesizing and assembling these transfecting agents, their formulation from well known components, their higher capability to deliver DNAs of larger sizes, their safety and ease of large scale production as reviewed by Wagner (15). Although non-viral carriers have poor transfection efficiency in comparison with viral vectors, they have received considerable attention after the formulation of Lipofectin® by Felgner and his research group (16) in 1987, which encouraged many other laboratories to develop non-viral gene delivery formulations. This research field continues to show promise for the improved efficiency of delivering DNA, but still there is a need for a better understanding of the mechanism of gene delivery to targeted cells (17) and detailed understanding of the barriers for non-viral gene delivery to achieve efficient formulation. These barriers that hinder the delivery of DNA to its physical site of action (the nucleus) for transcription and subsequent translation have been summarized (Figs. 1 and 2) as follows:

1. DNA condensation
2. Cell targeting
3. Cell membrane entry
4. Endosomal escape
5. Nuclear entry
6. Decomplexation
7. Transcription and translation

A review of the literature covers these barriers in turn, and then the excipients used in non-viral DNA formulation are critically surveyed.

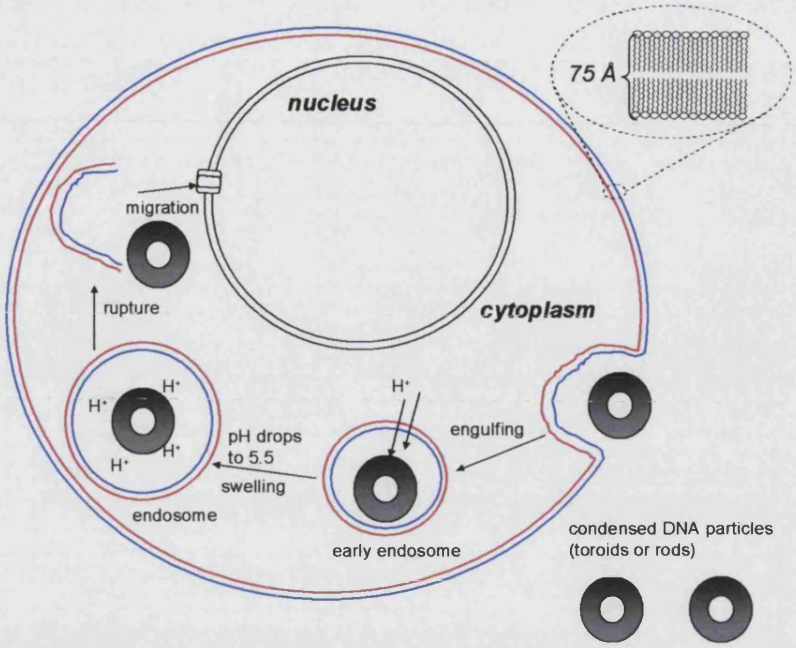


Figure 1. Steps in the process of non-viral gene therapy by endocytosis showing the barriers to DNA nanoparticles. This process goes from the formation of the DNA-polycation complex to protein synthesis which includes complex formation between the DNA and cationic polymer or lipid that leads to condensation of DNA into nanoparticles in the form of rods or toroids. According to the results reported by Böttcher et al. (18) using cryo-electron microscopy, the dimensions of toroids produced by condensation of pEG*lacZ* condensed with spermine was between 15-40 nm for the central hole, the external diameter from 50-110 nm and the thickness of the toroid from 11-28 nm. The calculated hollow volume was from 0.8×10^4 to 1.7×10^5 nm^3. Cell membrane entry is thought to be mediated by cationic substances which interact with DNA. This interaction causes adsorptive endocytosis and internalization of the complex. The internalized material is fused with early endosome, and then leads this process to sorting to the late endosomal compartment. At this stage, the DNA complex should escape the endosomal vesicle before the later stage of the lysosome where the DNA is degraded.

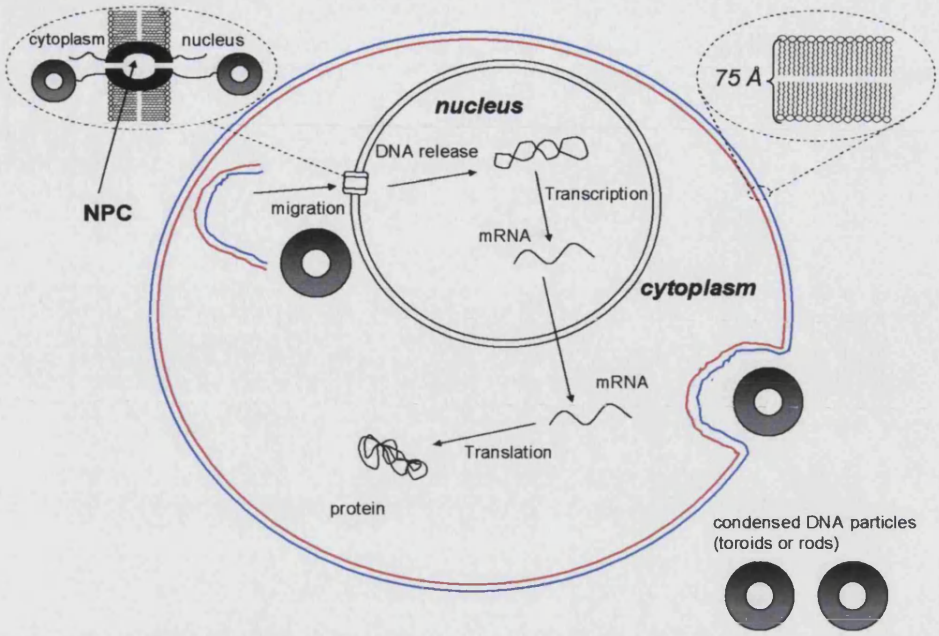


Figure 2. The steps in the process of non-viral gene therapy after endosomal escape that is considered to be the first barrier after cellular internalization of the nanoparticles. The DNA either complexed or dissociated from the condensing agent should finds its way (as DNA is subject to hydrolysis by DNase in the cytosol) to the nucleus. Also, the DNA should cross the nuclear membrane which thought to be occurring through the nuclear pore complex (NPC) or by direct association with the chromatin during mitosis. After nuclear entry, the DNA should successfully be able to give the desired protein through transcription and translation processes, e.g. access of transcription factors may be affected by the dissociation rate of the DNA complex.

Literature review

1. DNA condensation

For drug formulators, it is difficult to deliver a drug molecule of 3.3 kDa molecular weight carrying 10 negative charges, but in the case of the prodrug DNA, a 5 kbp plasmid has a molecular weight of about 3.3 MegaDa and carries 10,000 negative charges. Each base pair (bp) is defined as 660 Da (5) for an average/random DNA duplex sequence. So the first key step in gene delivery is DNA condensation into a nanoparticle form through masking the negative charges of the phosphate backbone. This titration with a cationic lipid or polymer causes alleviation of charge

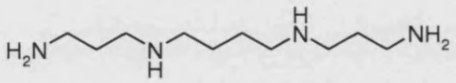
repulsion between remote phosphates along the DNA helix leading to collapse into a more compact structure. This is a phase transition from extended conformation to a compact structure that facilitates cell entry (19). This phase transition is favoured when about 90 % of the negative charges along the phosphate backbone are neutralized (20-22) based on the counter ion condensation theory by Manning (23-25).

The use of an efficient carrier for DNA is considered to be a determinant factor for the successful application of gene therapy. This carrier is responsible for the complex process of gene delivery to the nucleus (26). The ability of multivalent cations such as polyamines to condense DNA into rods (27) or toroids (28, 29) has provided DNA in a nanoparticle form suitable for gene delivery. Cationic lipids and polymers have the ability to condense DNA into particles that can be readily endocytosed by cultured cells. The formed nanoparticles and their size are essential for the development of gene delivery vehicles (30, 31). In addition, the cationic lipids and polymers increase the stability of DNA in serum (32).

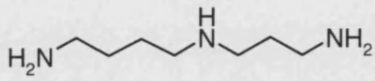
DNA is condensed by naturally occurring positively charged polyamines spermine and spermidine (Fig. 3) working together with the basic proteins histones (21). Histones are relatively small, highly basic (i.e. at physiological pH 7.4 they carry many positive charges) proteins with molecular weights in the range of 10 to 20 kDa (33). The five major types of histone chains are: H1, H2A, H2B, H3 and H4 differentiated on the basis of their relative content of lysine and arginine (Fig. 3). The amino acid sequences of four histones (H2A, H2B, H3 and H4), from a wide variety of organisms, are similar among different species (33). The histones appear to be involved in the supercoiling of DNA in eukaryotic chromosomes through their recurring positive charges, due to the presence of a high ratio of positively charged (basic) amino acids lysine and arginine (Fig. 3) that leads to the formation of electrostatic associations with the negatively charged phosphate groups of DNA, which renders the DNA more stable and flexible (33). The double helix of DNA, together with the associated histones, is supercoiled and folded back and forth many times in the chromatin fibres. Also, polymers of histidine and the non-protein basic amino acid ornithine (Fig. 3) have been used for DNA condensation (34-38).

There are few studies that utilized histones in the design of non-viral gene therapy. H1 and the cationic lipid DOSPER was formulated for the investigation of luciferase gene (pCMV Luc) delivery in ECV 304 human endothelial cells (39). In a different investigation, Hoganson et al. (40) showed that ligand-mediated endocytosis can specifically deliver DNA to the cells that have these specific receptors in-vitro. They targeted FGF receptor-bearing cells by the ligand fibroblast growth factor (FGF2) with DNA encoding saporin. Saporin is a potent ribosomal inactivating protein. In addition, FGF2 was also used in their study with DNA encoding the conditionally catatonic herpes simplex virus thymidine kinase, a protein that can kill cells by activating the prodrug ganciclovir. However, when histone H1, a ligand that binds to cell surface heparan sulfate proteolysis ("low-affinity" FGF receptors), was used to deliver DNA encoding thymidine kinase, no ganciclovir sensitivity was observed (40).

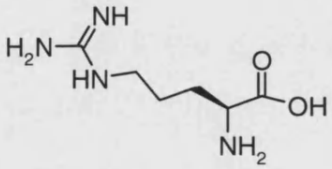
Spermine



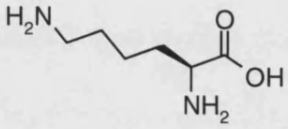
Spermidine



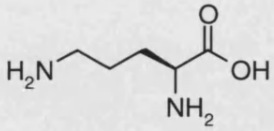
Arginine (Arg, R)



Lysine (Lys, K)



Ornithine (Orn, O)



Histidine (His, H)

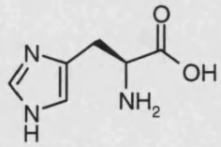


Figure 3. Spermine, spermidine and the positively charged amino acids arginine, lysine, ornithine and histidine.

Chemically modified histones were also investigated in gene delivery formulations. Galactosylated histone, a ligand to the asialoglycoprotein receptors, was synthesized and constructed in an Epstein-Barr virus (EBV) based expression vector to improve levels of the human cytokine interleukin-2 (IL-2) cDNA gene expression through targeted ligand receptor mediated endocytosis into hepatoma (HepG2) cells via the hepatocyte cell surface receptors (41). Also, H1 was conjugated with nuclear localization signal (NLS) for DNA binding and formulated with (dioleoyl phosphatidylethanolamine (DOPE) or phosphatidylserine (PS)) (42) as liposomal formulation.

Déas et al. (43) modified their vector (plasmid DNA with antibody) by introducing non-covalent binding between the antibody and DNA, allowing the possibility to introduce different important molecules. The non-covalent association was achieved with neutravidin and biotinylated components: (1) biotinylated antibodies; (2) a biotinylated hemagglutinin fusogenic peptide of influenza virus to favour endosomal escape; and (3) biotinylated histone H1 to condense, protect, and associate DNA to the complex.

DNA interaction studies with polycations showed the condensation of DNA to nanoparticles with different structures and different particle sizes that affect the delivery of DNA to the cells. The investigation of DNA condensation into rods and toroids, as in most vertebrate sperm cells (44), has attracted many research laboratories to thoroughly study the structure of DNA toroids. Studies on DNA toroids have revealed different models for the organization of DNA within toroids: the circumferential DNA wrapping (spool) model (18, 28), the constant radius of curvature model (45), and the latter model assumed by Hud and Downing (46). Also, detection and characterization of the condensed DNA nanoparticles using polycations showed a great interest by several laboratories (see Table 1). In addition, investigations using hexamine cobalt (III) to control the size of the condensed DNA particles revealed that the size of the initial nucleation loop in toroid formation has a major effect on the toroid size, also the ionic strength of the solution will affect the thickness of the formed toroids (47).

Table 1. DNA condensing agents and their application for nanoparticle formation and gene delivery

Carrier	DNA	Particle size (nm)	Method	Notes	Ref.
Spermidine	T7	45-130	EM or DLS	90 % have toroid shape when stained with uranyl acetate, while unstained have 10 % toroids, outer diameter : 75-97 nm inner diameter: 28.7-38.5 nm, collapse of DNA is a result of 89-90 % neutralization of the total phosphate charges	(20, 21, 48)
Spermine and spermine analogues	λ-phage, pEG*lacZ*, pSFiSVneo. pSfiSV19 or pCISfi-γIFN	50-130	SFM, DLS or EM	The distribution of positive charges along the polyamines backbone plays a major role in condensation of DNA.	(18, 49)
Reducible lipopolyamine analogue	pCMV-Luc	100	DLS	Over +/- charge ratio of 4 gives particles about 100nm, while at charge ratio between 1 and 3 very large particles were detected	(50)
Cationic silanes	pBR322	40	AFM	At lower cationic silanes concs. there is a flower and sausage shaped structure condensates, at higher concs. toroids and rods are formed	(51)
Cobalt hexamine	λ-phage, polynucleo-tides	Outer: 95-185 hole, 35-85	EM	Diameter of toroids formed may be affected by polylysine during preparation. Also, the volume of toroid and the amount of DNA /toroid can be calculated	(46, 47, 52)
Peptide and peptide conjugates	pCMV-β-gal, pCMVLuc	20-700	PCS, DLS	Short peptides with cationic lipids improve both condensation and transfection	(53, 54)
Chitosan and chitosan derivatives	pcRELuc, pEGFP1, pCMVCA,	27-750	DLS, TEM, AFM	Particle size decreased as the charge ratio increased	(55, 56)

Cont. **Table 1.** DNA condensing agents and their application for nanoparticle formation and gene delivery

Carrier	DNA	Particle size (nm)	Method	Notes	Ref.
Biodegradable polymers	25-mer ODN (Phosphoro-thioates), pGL3, pBR322, pSV-β-gal	70-400	PCS, TEM, DLS, AFM	Nanoparticles < 100nm showed higher transfection than larger nanoparticles	(57-59)
Thermo sensitive Polymers	pCMV-*LacZ*	150-200	DLS	Zeta potential can also be used as a characteristic to predict the behaviour of this type of co-polymer/plasmid complexes in transfection	(60)
Cholesterol Derivatives-	pSV2CAT	200-400 400-1400 >2000	AFM	Zeta potential is important factor for controlling transfection	(61)
DC-Chol – non-ionic surfactants	p CMVLuc	Tween 384 Span 1365 Brij 1937	DLS	Addition of non-ionic surfactant prevent the formation of large aggregates	(62)
Cationic liposomes (Cationic Cholesterol-DOPE-protamine)	pGL3	150-200	AFM	Addition of protamine facilitates the internalization of DNA complex to the nucleus	(63)
Cationic liposomes (DOTMA-DOPE)	pSV2-CAT	~ 250	LLS	The first NVGT formulation	(64)
Liposome-HK-Polymer	PCI-Luc	40-100	DLS	The ratio of His to Lys in the co-polymer affects transfection efficiency	(34)
Gelatine	pcRELuc	200-750	Differential interference contrast microscope	Nanospheres were synthesized by salt-induced complex coacervation	(65)

2. Cell targeting

Several experiments were applied to link a DNA complex with a targeting ligand in order to improve nonspecific binding of positively charged DNA nanoparticles to cells with a more specific interaction. This specific binding will lead to increase in the efficiency of transfection and reduction of the toxicity of the vector where DNA will be internalized by receptor-mediated endocytosis more efficiently to specific organs or cells that contain receptors for the target ligand. To utilize this improvement by targeting ligands, a number of gene delivery systems have been formulated to include targeting ligands. These ligands were derived from several classes including carbohydrates, vitamins, peptides and antibodies.

From the carbohydrate class, galactose and mannose were investigated for cell-specific receptor mediated endocytosis in gene delivery using mannosylated poly-L-lysine and galactosylated poly-L-lysine (66-68). The asialoglycoprotein receptor on hepatocytes and the mannose receptor on macrophages and liver endothelial cells are the targets for galactosylated and mannosylated gene carriers (67, 69). Behr, Remy and coworkers designed lipopolyamine-DNA complexes with galactose (70) and galactosylated chitosan was formulated for hepatocyte targeting (71, 72). Galactosylated histone as a ligand was synthesized and constructed in a viral vector to improve levels of gene expression into hepatoma (HepG2) cells (41). Lactose was also utilized for hepatoma cell-targeting as lactose-poly(ethylene glycol)-grafted poly-L-lysine gene carrier, also lactose used for targeting cystic fibrosis (CF) airways that shows improved transfection and reduced cytotoxicity (73, 74).

Group-B vitamins were also utilized for targeting cancer cells, the folate receptor is a tumor marker over-expressed in most human tumors that was studied for targeting using folic acid (B9) (75-77). As cancer cells have high demand for Vitamin B12 taken up by receptor mediated endocytosis, this opens the possibility for Vitamin B12 to be used as a targeting ligand that leads to preferential uptake by cancer cells, invistigated by Grissom and co-workers (78, 79).

Among the peptides class of targeting ligands, the synthetic peptide arginine-glycine-aspartate (RGD) that was successfully introduced in the formulation of non-viral vectors for integrin receptor targeting (80, 81). Transferrin, a natural iron-delivery protein was also investigated for targeted gene delivery. Birnstiel, Wagner and co-workers was a leading group to investigate transferrin as targeting ligand to the transferrin receptor in various cell lines using a transferrin poly(L-lysine) conjugate as a carrier (82-84). Transferrin was also incorporated with cyclodextrins polymer-based gene delivery system. The non-viral system is formed by condensation of a cyclodextrin polycation with nucleic acid into nanoparticles that are surface-modified to display poly(ethylene glycol) (PEG) for increasing stability in biological fluids and transferrin for targeting of cancer cells that express transferrin receptor (85).

The ligand fibroblast growth factor (FGF2, a 155-amino acid) targeted FGF receptor-bearing cells that showed efficient ligand-mediated endocytosis (40, 86). A cell-binding ligand, the transactivator of HIV (TAT, sequence GRKKRRQRRRGYG) transcription peptide stimulated cellular uptake was incorporated in liposomal cationic lipid formulation and compared with DOTAP/DOPE liposomes and PEI (87). The assembled liposomal formulation showed 80-fold enhancement of transfection efficiency. HIV-TAT derived peptide show efficiency in targeting DNA and glycosaminoglycans for directed gene and drug delivery (88). An antisense gene targeting formulation to brain cancer cells include epidermal growth factor chimeric peptides (EGFR) that reduces the growth of EGFR-dependent gliomas (89).

Antibody-based gene delivery vector was designed for tumour cell targeting to the G250 renal cell carcinoma-associated antigen in tumour cells (43). Antibody-based gene targeting was designed for ovarian carcinoma cells using pegylated polyethylenimine (PEG-PEI) conjugated to the antigen binding fragment (Fab') of the OV-TL16 antibody directed to the OA3 surface antigen that is expressed by a majority of human ovarian carcinoma cell lines (90). The results revealed luciferase expression increased up to 80-fold compared to PEG-PEI and was even higher than that of PEI 25 kDa non-viral vectors. In addition, peptide nuclear localization signals (NLS) were successfully applied for DNA-nuclear targeting inside the cell after endosomal escape.

3. Cell membrane entry

The mammalian cell membrane is a semi-permeable membrane formed from a phospholipid bilayer and containing various integral proteins. The cell membrane selectively screens the entry of foreign matter to the cell and allows the transport of macromolecules by endocytosis. Neutralization of the negative charges on the DNA by polycations will improve the delivery of DNA through the cell membrane by masking the negative charges on DNA because of the presence of negative charges on both DNA and cell membrane that hinder the delivery of DNA alone. Also, the positively charged lipid complex will mediate transfection by fusion with the cell membrane and internalization by endocytosis and/or receptor mediated endocytosis (91).

4. Endosomal escape

The major intracellular barriers that face the delivery of DNA into the nucleus are located in the cytoplasm. Although the DNA complex is efficiently internalized in the cell, small fraction will be transferred to the nucleus and expressed (92). As the DNA complex is internalized, it is retained inside the early endosomes, then sorted to the late endosomes that are characterized by increased acidity (low pH value). At this stage, the efficient escape of DNA from the endosome is an important factor for successful gene delivery before further trafficking of the trapped DNA to the lysosomal compartment where the DNA is subjected to degradation by the nucleases found in the lysosomes (15).

The possible mechanisms for endosomal escape are:

1. Disruption of the negatively charged endosomal membrane through mixing with the cationic lipid vector (93).

2. Cationic polymer interaction with the negatively charged endosomal membrane. (94).

3. Proton sponge hypothesis, where a cationic polymer, such as polyethylenimine, has the ability of endosomal pH buffering that leads to

chloride ion influx together with water molecules that leads to membrane swelling and disruption. (95).

4. Membrane destabilising peptides, which includes fusogenic or lytic peptides, such as the basic peptide KALA (96), acidic peptide EGLA (15) or the uncharged peptides at neutral pH such as H5WYG (35) these peptides have the ability to disrupt the endosomal membrane and increase the release of DNA to the cytosol.

5. Compounds that act as lysosomotropic agents assist the escape of the endosome such as chloroquine that inhibit lysosomal processing and sucrose that cause endosomal compartment osmotic lysis, are used with the condensing agents to improve the efficiency of non-viral gene delivery formulations (97, 98).

5. Nuclear entry

One of the crucial steps for successful delivery of DNA into its site of action is crossing the nuclear membrane. The entry of macromolecules to the nucleus occurs through the nuclear pore complex (NPC) which allows a passive diffusion of molecules of up to 50 kDa molecular weight or about 10 nm in diameter, but in the case of larger molecules NPC is capable of actively transporting particles of about 25-50 MDa or 25 nm in diameter (99).

The utilization of nuclear localization signals (NLS) through their characteristics to target the macromolecules to the nucleus and increase the transfection efficiency have been applied (100). The other way for DNA entry to the nucleus is during mitosis where nuclear membrane is broken down so the DNA and macromolecules can easily gain access.

The first nuclear localization signal to be mapped in detail was characterized by Smith and colleagues in 1984 (101). The T-antigen nuclear localization signal is a seven amino acid sequence Pro-Lys-Lys-Lys-Arg-Lys-Val. Not only was this sequence necessary for the nuclear transport of T-antigen, but also its addition to

other, normally cytoplasmic, proteins was also sufficient to direct their accumulation in the nucleus (33).

The use of nuclear localization signals in non-viral formulation has been investigated with encouraging results. The signal PKKKRKVEDPYC, derived from SV40, was used to increase PEI- and Transfectam-mediated transfection significantly in terms of the amount of DNA required for in-vitro transfection (100). In addition, the yeast transcriptional activator, GAL4, has been shown both to bind DNA and separately act as a nuclear localization signal (102). Nuclear localisation signals are used as adjuvant in the gene delivery formulation to improve transfection efficiency (103). Noguchi et al. (63) promoted gene transfection of the DNA-protamine-liposome complex with the derivative (I) more significantly into the nucleus of the target cells using the nuclear localization signal of protamine.

Nakanishi et al. (104) compared the ability of various synthetic NLS peptides to carry large molecules into nuclei. They chemically modified bovine serum albumin (BSA, MW 70 kDa) or IgM (MW 970 kDa, diameter 33 nm) using these peptides, then estimated the propensity of these molecules to translocate into nuclei by injecting them into cytoplasm. Although many NLS can promote the nuclear transfer of BSA, only 34mer peptide from SV40 T-antigen could carry IgM into the nucleus, as reported (105). A shorter peptide (12-mer) containing NLS of T-antigen could carry BSA, but not IgM into the nucleus, suggesting that the longer peptide contains the additional information required to transfer large molecules into nuclei (105). However, the understanding of this difference in transport ability on a molecular basis still remains unclear.

6. Decomplexation

The dissociation of DNA from the condensing agent is an important step for the host transcription factors to initiate the transcription reaction. The decomplexation process may occur after endosomal escape in the cytoplasm (93) or after nuclear entry as indicated by Mikos and his group (106) using confocal images for tracking PEI/DNA complexes. Also, the kinetics of intracellular DNA complex dissociation are important factor for successful non-viral gene delivery process (107).

If the DNA dissociated from the condensing agent easily and happened in the early stages of cellular entry, this will lead to degradation of the delivered gene by DNase in the cell. On the other hand, if the dissociation rate is slow, this will hinder the accessibility of the host transcription factors to initiate the transcription reaction that leads to low or no level of protein expression.

7. Transcription and translation

Inside the nucleus, the next step for gene therapy is the transcription process which depends on the promoter strength and presence of enhancer in the vector which have an effect on the transfection efficiency (108). The successful transcription process leads to the translation and formation of the desired protein (the active form of the prodrug DNA). Kamiya et al. (107) designed a simple pharmacokinetics/ pharmacodynamics model for the expression of the delivered gene in the nucleus based on the general consideration that the total amount of mRNA depends on the amount of the exogenous DNA in the nucleus. They concluded from this model that the amount (efficiency) of DNA delivered to the nucleus is the only determinant for expression of the delivered gene.

Excipients used in non-viral DNA formulation

Traditionally, the process of drug design, development and preformulation starts with main focus on the design of the active chemical of interest (candidate drug) followed by the selection of the excipients. In the case of non-viral gene therapy, the key step is the selection of the suitable excipients (the vector) for the delivery of the prodrug (DNA or RNA) into its site of action. Due to the polyanionic nature and the size of the active ingredient, the efforts continue to design novel or improve existing non-viral gene delivery formulations in order to increase the stability of the drug (DNA), decrease the toxicity of the excipients and/or increase transfection efficiency. Also, as a result of the improvement in DNA delivery systems, the strategy of antisense oligonucleotide delivery to inhibit gene expression has been widely investigated (109, 110). Vectors used for non-viral gene formulation can be classified as cationic polymers or cationic lipids (5).

Cationic polymers

One of the leading cationic polymers that has been used as non-viral vector for gene delivery is polyethylenimine (PEI) (95). PEI, either linear or branched with different MWs (Fig. 4), possess a buffering capability that inhibits lysosomal enzyme activity by lowering proton concentration (proton sponge hypothesis) and presumably promoting the release of DNA that leads to an improvement in the transfection efficiency.

Branched polyethylenimine **Linear polyethylenimine**

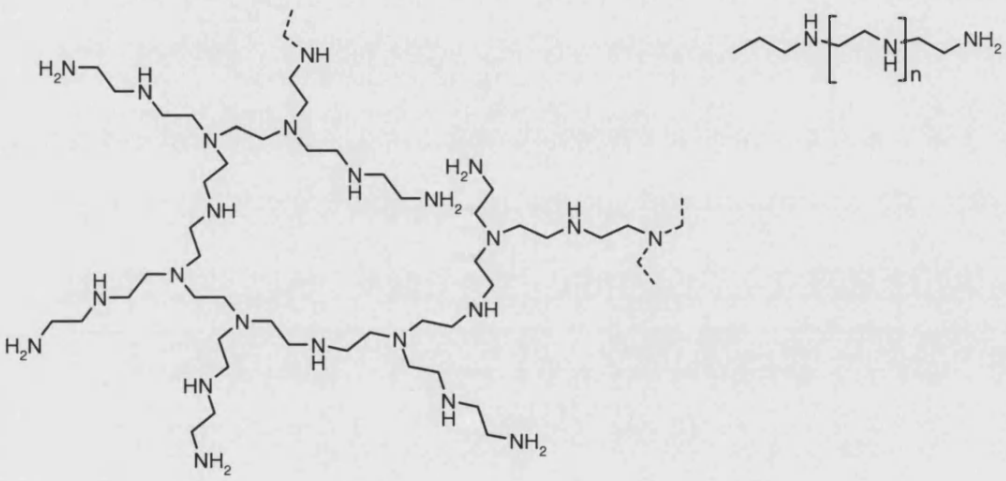


Figure 4. Branched and linear PEI.

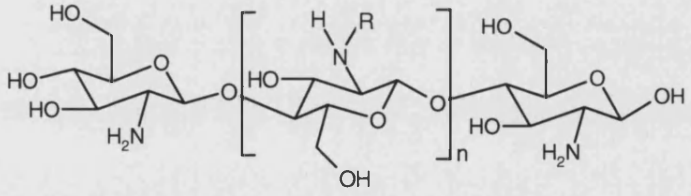
Polyethylenimines (PEI) have shown continued investigation to improve their cell viability and ability as non-viral gene delivery vectors utilising their endosomal escape efficiency that help the complex to escape the endosome and improve the transfection efficiency of the delivered gene (90, 111-115). In addition, PEI was used in conjugation with cationic lipids to utilise the characteristics of both vectors in one formula (116).

On the other hand, among cationic polymers, the second class of non-viral vectors, are the cationic polysaccharides (amino sugars) that have received increased focus as promising vectors for gene delivery utilising their well known advantage of

safety and long experience use of these compounds in pharmaceutical industry and drug formulations.

Chitosan (*N*-deacetylated chitin) is a cationic, linear biodegradable polysaccharide composed of β-(1,4)-linked glucosamine partly containing *N*-acetyl-glucosamine (Fig. 5). Chitosan is derived by deacetylation of naturally occurring chitin in crab and shrimp shells. The pK_a value of the amino groups of the deacetylated moieties is 6.5 (117). Mumper et al. (118) were the first research group to use chitosan as condensing agent in non-viral gene therapy. The biocompatibility and safety (119, 120) of chitosan renders it potentially useful for gene delivery (121-123).

Chitosan was evaluated for its ability to transfect different cell lines in comparison with Lipofectamine™ (65, 124), Lipofectin® (72), but chitosan used in this paper was coupled with galactose for hepatocyte targeting (72), poly-L-lysine (125), PEI (126), and in-vivo administration with endosomolytic peptide GM225.1 (127), these results showed the ability of chitosan to be used as safe DNA carrier.



R = H glucosamine
R = $COCH_3$ *N*-acetyl glucosamine

Figure 5. Model structure of a typical, idealised chitosan, where n might be 200-3000 giving a molecular weight from 40-600 kDa.

Other laboratories tried to chemically modify chitosan in order to increase the transfection efficiency, enhance cell specificity and/or increase DNA complex stability (128-130). Chitosan was also chemically modified with hydrophobic cholesterol groups to prepare chitosan-based polymeric amphiphiles (131). With a similar strategy, Yoo et al. (132) prepared a hydrophobically modified glycol chitosan with 5β-cholanic acid (Fig. 6) based upon their earlier studies (133, 134). The in-

vivo results revealed higher transfection efficiency over naked DNA. Chitosan microspheres were used to encapsulate two plasmids without affecting their structural and functional integrity that leads to sustained and high protein production (135). Chitosan was also combined with adenoviral vectors to increase the adenoviral infectivity to mammalian cells in-vitro and in-vivo (136, 137).

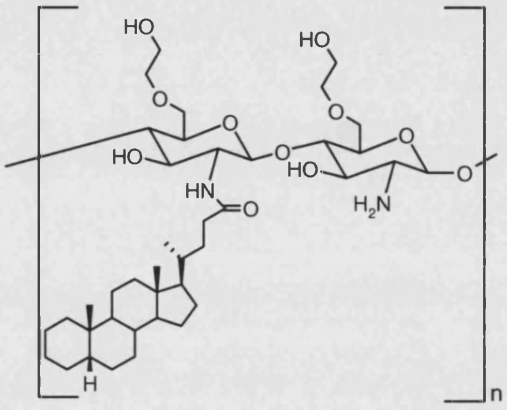


Figure 6. 5β-Cholanic acid hydrophobically modified glycol chitosan.

To show the mechanism of gene delivery by chitosan, Ishii et al. (138) analysed the transfection mechanism of plasmid/chitosan complexes as well as the relationship between transfection activity and cell uptake by using fluorescein isothiocyanate-labelled plasmid and Texas Red-labelled chitosan. They found that plasmid/chitosan complexes most likely condense to form large aggregates (5-8 μm), which absorb to the cell surface. After this, plasmid/chitosan complexes are endocytosed, and possibly released from endosomes due to swelling of lysosomal compartment in addition to swelling of plasmid/chitosan complex, causing the endosome to rupture. Ultimately, complexes were also observed to accumulate in the nucleus using a confocal laser scanning microscope.

Chitosan was also investigated for its interaction with a lipid bilayer (didodecyl dimethylammonium bromide, DDAB) (139). The results showed that the low MW chitosan could disrupt the lipid bilayer, and the effect seemed to be in a concentration-dependent manner. Also, the modification of chitosan using alkyl bromide leads to the formation of DNA complexes at lower charge ratios than the unmodified chitosan (140).

The long-term stability of chitosan-based polyplex solutions (25mM sodium acetate buffer, pH 5.5) was investigated at different temperatures (4°C, 25°C, 45°C) over a period of up to 1 year (141). The results show that, one year of storage at 4°C did not result in any major changes in the properties of the polyplexes. At 25°C there were minor changes in the physicochemical characteristics of the polyplexes (particle size and zeta potential), and the in-vitro transfection efficiency was reduced at 1 year of storage. Storage at 45°C altered both the in-vitro transfection efficiency and the physicochemical properties of the polyplexes after a short time.

Novel non-viral vectors were also synthesized from a carbohydrate co-monomer (esterified D-glucaric acid) within a PEI-like backbone and also, a polymer of esterified D-glucaric acid with diethylenetriamine, triethylenetetraamine, tetraethylenepentamine, and pentaethylenehexamine (Fig. 7) by Reineke and colleagues (142, 143), these 2.2.2-mers have previously been purified and conjugated (144, 145). With the same concept, the carbohydrates D-trehalose and β-cyclodextrin (CD) were used within the polycation backbone to condense DNA into particles that can be readily endocytosed by cultured cells (146). Cyclodextrins continue to be promising non-toxic non-viral vectors. Their structural effects were investigated on both gene delivery and conjugation with polyamidoamine dendrimers (146-149). β-Cyclodextrins were modified to form cationic amphiphiles and Polycationic (polyamino) β-cyclodextrins with different cationic groups (amino, pyridylamino or butylimidazole) that were used for efficient DNA condensation and transfection (150, 151). Cyclodextrins were also conjugated with the targeting ligand transferrin for tumour cells and polyethylene-glycol (PEG) to improve stability in biological fluids (85). Azzam et al. (152) have synthesized spermine conjugated with different molecular weight dextrans as gene delivery vectors.

Also, Zhang et al. (153) used the sugar-based non-ionic surfactant, octylpolyglucosides (C(8)APG), which caused DNA conformational collapse in aqueous solution as the concentration increased. They have used UV and CD to determine the change of DNA second-order structure to confirm the above results.

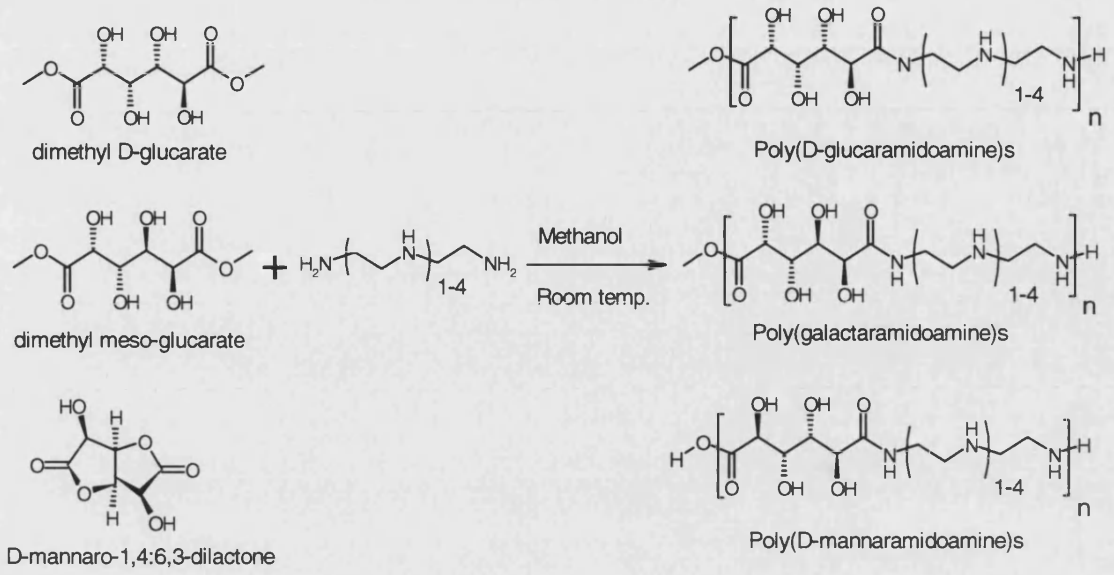


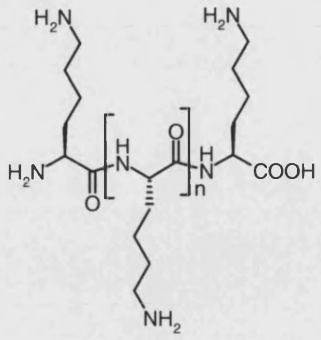
Figure 7. Synthesis of poly(glycoamidoamine)s (143).

Cationic peptides

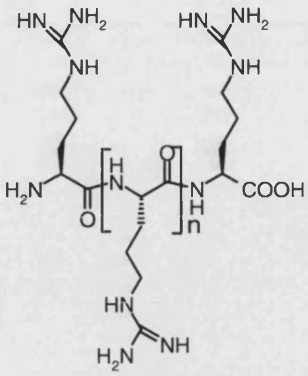
Cationic peptides that include the positively charged amino acids lysine, ornithine and arginine are utilized in the design of non-viral delivery vectors. Arginine has a pK_a value of 12.5 and both lysine and ornithine have similar pK_a values of 10.8. Arginine and lysine are the major constituents of histones (responsible for DNA condensation in the nucleus) and nuclear localisation signals of the delivered proteins from the cytoplasm to the nucleus where they have been used as vectors for DNA delivery (154, 155).

Some research groups are interested in the use of the basic amino acid polymers such as poly-L-ornithine, poly-L-lysine and poly-L-arginine (Fig. 8) that carry multiple positive charges at physiological pH in DNA condensation and gene delivery (37, 38). Also, Wender, Rothbard and their co-workers investigated the role of arginine oligomers as drug transporters for their ability to cross the biological barriers (156, 157). Wender, Siprashvili and others also reported (158) that flanking a core of consecutive arginines with cysteines improve the efficiency of the delivered gene not only in-vitro, but these new peptides also increased gene expression in both murine and human tissue in-vivo.

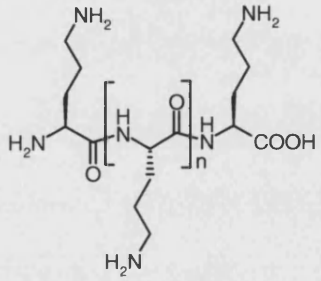
Poly-L-lysine



Poly-L-arginine



Poly-L-ornithine



Poly-L-histidine

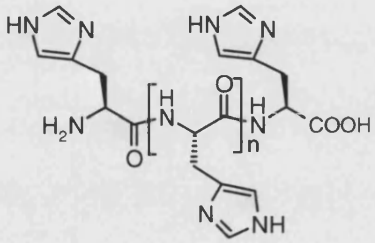


Figure 8. Poly-L-lysine, poly-L-arginine, poly-L-ornithine and poly-L-histidine.

Chamarthy et al. (36) examined the binding, toxicity and gene delivery efficiency of three low molecular weight peptides, K-16, K10H6, and O10H6, derived from the peptides poly-L-ornithine, poly-L-lysine and poly-L-histidine (Fig. 8). They concluded that the in-vitro transfection with DNA complexed with cationic peptide O10H6 resulted in the highest level of gene expression and lower toxicity in comparison with K-16 and K10H6 cationic peptides.

Poly-L-lysine and poly-L-ornithine (Fig. 8) were converted into amphiphilic colloid by incorporation of a distearoylphosphatidylethanolamine poly(ethylene glycol)-galactosamine conjugate (with the galactosamine unit at the distal end of the poly(ethylene glycol) moiety) into the polyamino acid formulations forming molecules that reduced their toxicity (159).

On the other hand, the efficient entry of viruses and toxins into cells and the action of antibacterial peptides have directed some laboratories to test membrane-active agents as components to enhance transgenic expression in non-viral gene delivery. Wagner (15, 160) and Szoka (161) and their respective research groups have used synthetic peptides derived from the N-terminus of influenza virus haemagglutinin, for example INF6, INF7, INFA, INF10, E5CA and the artificial amphipathic peptides such as EGLA-I (sequence listed in Table 2) and the rhinovirus HRV2 VP-1 protein. These peptides may have specificity for endosomal pH due to acidic residues (glutamic and aspartic acids) aligning on one side of an amphipathic helix. At neutral pH, the negatively charged carboxylic groups destabilize the alpha-helical structure; acidification of the carboxylic groups shifts the equilibrium towards amphipathic helical structure, which promotes multimerization of peptides and/or membrane interaction.

The pH specificity can be enhanced by the introduction of additional glutamic acids into the peptide sequence (15). Additionally, the incorporation of a fusogenic peptide JTS-1 (Table 2), into either a DNA-liposome or a DNA liposome-polylysine-complex gave a 6-14-fold improvement in the expression level of luciferase (162). Also, amphiphilic basic peptides such as melittin (peptide from bee venom) (163), K5 the cationic counterpart of E5 (164) and KALA the lysyl counterpart of GALA (96) (Table 2) exhibit efficient membrane fusion and permeabilization activities at both neutral and acidic pH. KALA, by its cationic nature, can form a complex with DNA, gave a 100-fold higher luciferase expression level than the polylysine-DNA complex in CV-1 cells. KALA itself was used for both DNA condensation and membrane destabilization, and thus induced increase in transfection efficiency (165). However, the KALA peptide cannot transfect primary airway epithelial cells (108).

Pichon et al. (35) designed uncharged peptides at neutral pH that neither interact with the plasma membrane nor with serum proteins, but become fusogenic in endosomes and this is achieved by making use of histidine. The imidazole group in histidine (Fig. 8) has a pK_a value of 6.0 and in the acidic environment of the endosomal compartment (pH 5.5) induces a buffering effect due to imidazole protonation leads to destabilization of the endosome that leads to the escape of the

delivered gene from the endosomal compartment before the lysosomal stage that will degrade DNA.

H5WYG peptide (GLFHAIAHFIHGGWHGLIHGWYG) was designed as analogous to the N-terminal segment of the HA-2 subunit of the influenza virus hemagglutinin (GLFGAIAGFIEGGWTGMIDGWYG) in which G-4, G-8, E-11, T-15 and D-19 were replaced by histidyl residues and M-17 by a leucyl residue (166). Other peptides containing histidines together with other peptides that have application in non-viral gene therapy are presented in Table 2. Other lysosomal enzyme inhibitors that assist the escape of the endosome, such as catapsin D and chloroquine, are used to improve the efficiency of non-viral gene delivery formulations (108).

Table 2. Representative membrane-active peptides that have gene delivery applications.

Name	Sequence	Reference
Anionic peptides		
INF6	GLFGAIAGFIENGWEGMIDGWYG	(160)
INF7	GLFEAIEGFIENGWEGMIDGWYG	(160)
E5CA	GLFEAIAEFIEGWEGLIEGCA	(167)
JTS-1	GLFEALLELLESLWELLLEA	(167)
GALA	WEAALAEALAEALAEHLAEALAEALEALAA	(161)
Cationic peptides		
melittin	CIGAVLKVLTTGLPALISWIKRKRQQ	(163)
KALA	WEAKLAKALAKALAKHLAKALAKALKACEA	(96)
K5	GLFKAIAKFIKGGWKGLIKG	(164)
Histidine peptides		
H5WYG	GLFHAIAHFIHGGWHGLIHGWYG	(35)
Histatin5	DSHAKRHHGYKRKFHEKHHSHRGY	(168)
Sea Urchin B18	LGLLLRHLRHHSNLLANI	(169)
LAH_4	KKALLALALHHLAHLALHLALALKKA	(170)

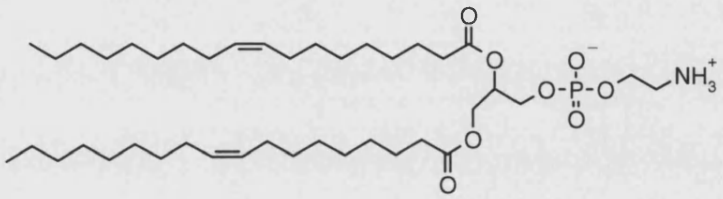
Cationic lipids

Cationic lipids are the most studied among the non-viral vectors as indicated by their encouraging transfection results over cationic polymer and naked DNA strategies. Since the design and formulation of Lipofectin by Felgner and co-workers in 1987, the focus on non-viral vectors in general, particularly on cationic lipids for DNA delivery, has shown a remarkable increase worldwide in comparison with the attention to viral vectors. The focus on cationic lipids as non-viral vectors was also clear from the 10 reviews in one issue of the journal Current Medicinal Chemistry (Volume 10, Number 14, July 2003) as well as other reviews (171, 172) dealing with cationic lipids and their roles in gene delivery. In non-viral gene therapy, cationic lipids are either in the form of liposomal or non-liposomal formulations. Liposomal formulations are composed of two types of lipid, a cationic lipid for DNA condensation and a helper lipid to improve both the stability and the transfection efficiency of the formed liposomes. Most commonly used helper lipids are dioleoyl phosphatidylethanolamine (DOPE), dioleoyl phosphatidylcholine (DOPC) or cholesterol (Fig. 9). A liposomal formulation of the cationic lipid DDAB and DOPE interacted faster with model cell membrane than the same liposomal formulation containing cholesterol as the helper lipid (173).

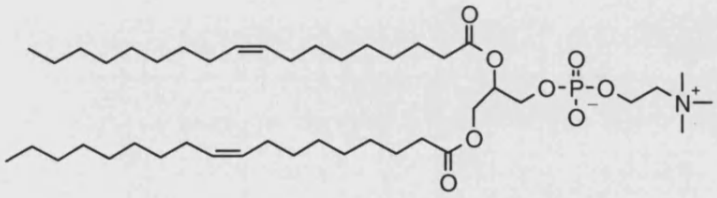
Lipofectin was formulated as a liposomal formulation, a well studied drug delivery system, that contains 1/1 w/w cationic lipid, *N*-[1-(2,3-dioleyloxy) propyl]-*N,N,N*-trimethylammonium chloride (DOTMA), and the helper lipid DOPE, respectively (16). Neutral phospholipid DOPE is used in liposomal formulation to increase transfection efficiency as it has a membrane destruction promoting ability in acidic conditions (108). Following the formulation of DOTMA, efforts continue to improve the transfection efficiency of cationic lipid formulations by modifying the structure of the cationic lipid. These efforts led to the synthesis of the monovalent cationic lipids, DOTAP (*N*-[1-(2,3-dioleoyloxy) propyl]-*N,N,N*-trimethyl ammonium (174) and GAP-DLRIE [*N*-(3-aminopropyl)-*N,N*-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide] (175) (Fig. 10). Heyes et al. (176) investigated the change in degree of unsaturation of the monovalent cationic lipid (1,2-distearyloxy-*N,N*-dimethyl-3-aminopropane (DSDMA). They concluded that increase in the degree of unsaturation improves gene silencing (siRNA delivery) efficiency.

A different strategy is to increase the number of the positive charges of the cationic lipid in the liposomes. These efforts presented spermine lipid conjugates, 2,3-dioleyloxy-*N*-[2 (sperminecarboxamido) ethyl]-*N,N*-dimethyl-1-propanaminium (DOSPA) that carry 4 positive charges at pH 7.2, and 1,3-di-oleoyloxy-2-(6-carboxy-spermyl)-propylamid (DOSPER) (177) with 3 positive charges at pH 7.2 were synthesized (Fig. 10). Lipofectamine (Invitrogen) is a commercially available liposomal formulation contains 3/1 w/w cationic lipid, DOSPA and the neutral lipid DOPE, respectively.

DOPE



DOPC



Cholesterol

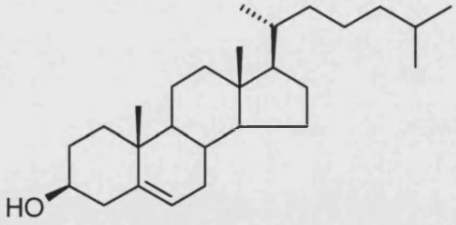
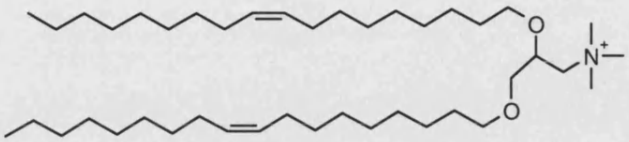


Figure 9. Neutral helper lipids DOPE, DOPC and cholesterol used in liposomal non-viral gene formulations.

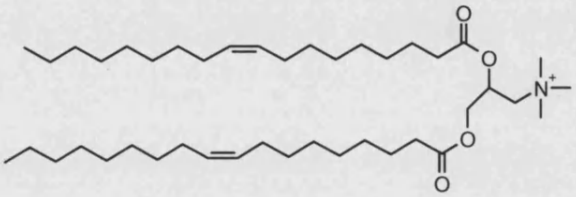
Safinya, Ewert and co-workers synthesized and characterized MVL5 a multivalent cationic lipid with a double-branched head-group structure (Fig. 10) (178). They formulated the cationic lipid with DOPC. The lipid head-group is attached to an unsaturated *cis*-C18 double chain hydrophobic moiety based on 3,4-dihydroxybenzoic acid. In their study, MVL5/DOPC liposomal formulation achieved

higher transfection efficiency when compared with the monovalent cationic lipid DOTAP/DOPC system (178).

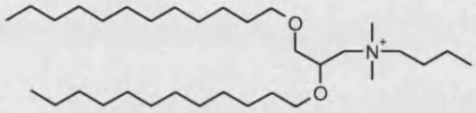
DOTMA



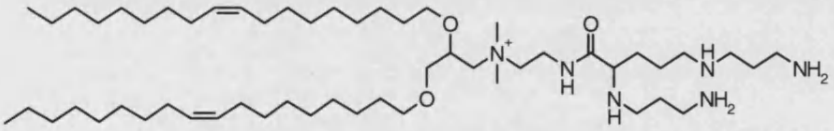
DOTAP



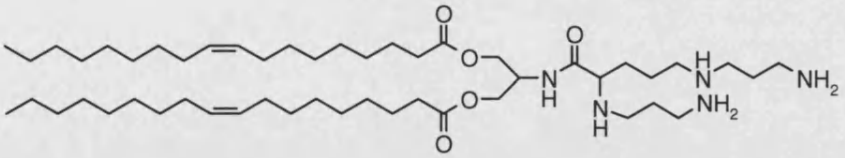
GAP-DLRIE



DOSPA



DOSPER



MVL5

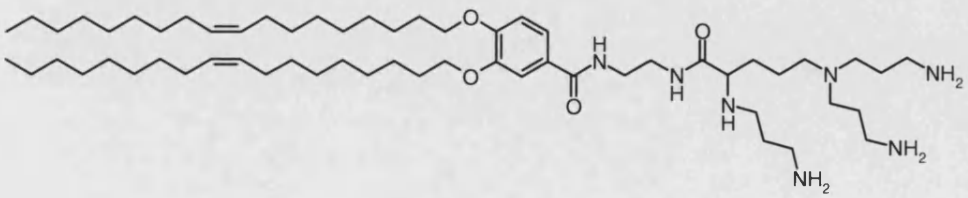


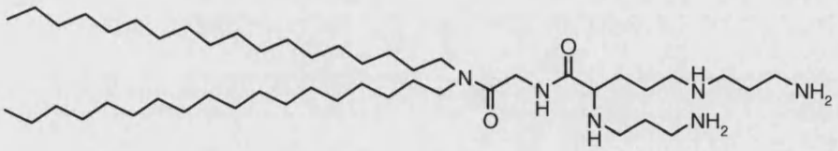
Figure 10. Monovalent and multivalent cationic lipids used as non-viral gene delivery vectors in the form of liposomal formulations with the neutral helper lipids DOPE, DOPC and cholesterol.

On the other hand, some research groups including us are focusing on the design, synthesis and formulation of novel simple non-liposomal cationic lipids. The lipopolyamine dioctadecylamidoglycylspermine DOGS (Fig. 11), commercialized as Transfectam (Promega), was the first non-liposomal formulation to be synthesized by Behr and co-workers (179). In the same work, they synthesized the lipospermine dipalmitoyl phosphatidylethanolamidospermine (DPPES) (Fig. 11) (179). Also, Dauty and Behr (180) investigated the control of the size of condensed DNA particles through monodisperse lipoplex population of 35-nm particles. They achieved this size through condensation of DNA molecules by cationic thiol-detergent, tetradecane-cysteine-ornithine (C14-COrn) detergent. The stability of DNA complexes with two cysteine surfactants (guanidinocysteine *N*-decylamide, C10-CG, and ornithinyl-cysteinyl-tetradecylamide, C14-CO) was investigated. These complexes were able to convert themselves, via oxidative dimerization, into cationic cystine lipids (181).

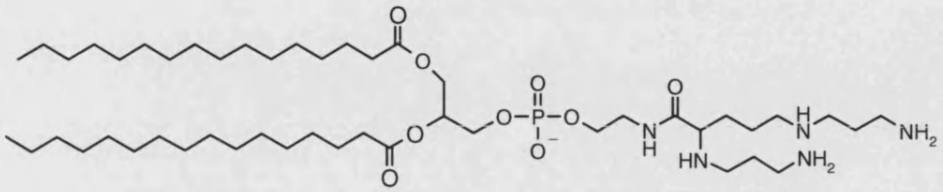
Scherman, Byk and co-workers synthesized the lipopolyamine RPR-120535 (182), they also introduced a reduction sensitive disulfide bridge between the lipopolyamine and side chain entity by adding a third hydrophobic chain, RPR132688 (Fig. 11), that improves the transfection efficiency over the non reducible RPR-120535 (183). Vierling and co-workers have synthesized fluorinated lipospermines that have hydrophobic and lipophobic chains (184, 185). They synthesized close analogues to DOGS, for example [F4C11][C14]-GS and [F4C11][F8C2]-GS (Fig. 11), to improve the transfection efficiency of non-viral vectors. Camilleri, Kirby and co-workers, have designed a series of cationic Gemini surfactants, for example GSC103 (Fig. 11) and GS11, that shows an increase in the level of gene expression compared to commercially available non-viral formulations (186, 187).

Gao and Huang, synthesized the first cholesterol based cationic lipid, (3β[*N*-(*N'*,*N'*-dimethylaminoethane) carbamoyl] cholesterol) DC-Chol (188) (Fig. 12). Cholesterol derivatives can be used as liposomal formulation with DOPE or as non-liposomal formulation without the use of DOPE. In an effort to modify the structure of cationic cholesterol lipids to improve the transfection efficiency, Lehn and co-workers have synthesized guanidinium cholesterol cationic lipids, bis-guanidinium spermidine cholesterol (BSGC) and bis-guanidinium tren cholesterol (BGTC) (Fig. 12) by replacing spermidine in BSGC with tris(2-aminoethyl) amine (tren) (189).

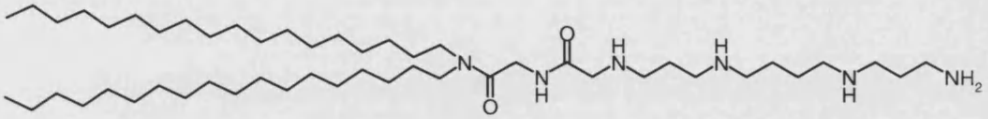
DOGS



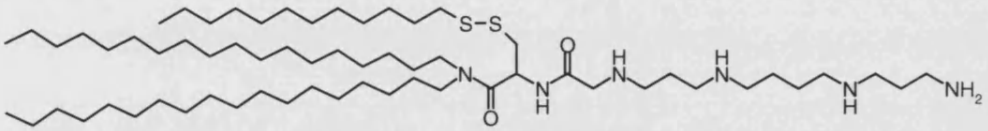
DPPES



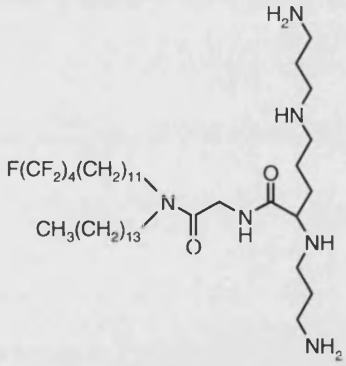
RPR-120535



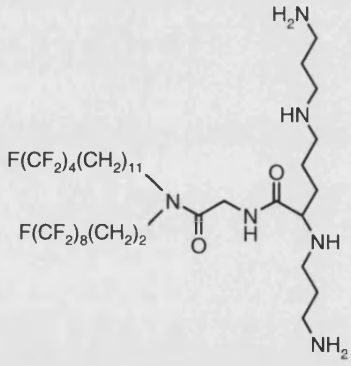
RPR-132688



[F4C11][C14]-GS



[F4C11][F8C2]-GS



GSC103

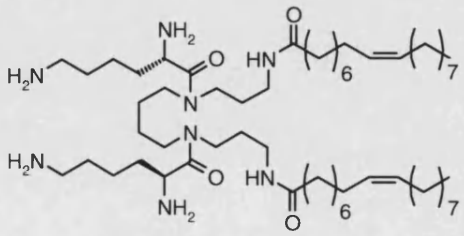
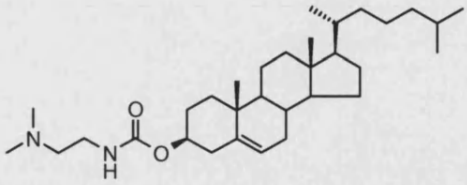
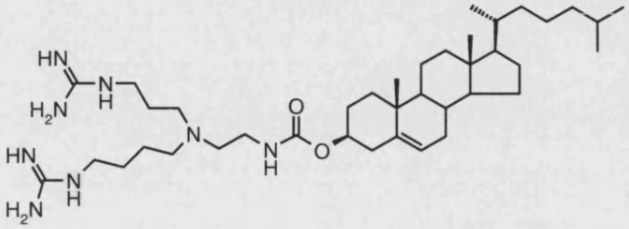


Figure 11. Spermine based lipopolyamines that can be used as non-liposomal formulations for non-viral gene delivery.

DC-Chol



BGSC



BGTC

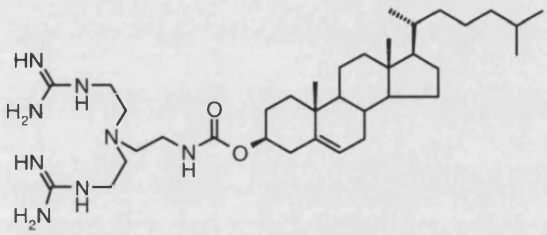


Figure 12. DC-Chol and bis-guanidinium-spermidine-cholesterol (BGSC) and bis-guanidinium tren-cholesterol BGTC. TREN, tris (2-aminoethyl) amine.

BGTC shows a high efficiency when used as a non-liposomal cationic lipid. In addition, both BGTC and BGSC are efficient when used as liposomal formulation in combination with DOPE in a variety of cell lines (189). Thiocholesterol-based liposomal cationic lipid nano-lipoparticles (NLP) were synthesized in which a cell-binding ligand (TAT peptide) was incorporated with a negatively charged (glutathione) or zwitterionic (cysteine) reducing agent to the particle surface and compared with DOTAP/DOPE liposomes and PEI (87). This assembled NLP, with a zwitterionic surface, gave a larger transfection yield than the cationic thiocholesterol-based cationic liposomes at all concentrations tested, but still these are liposome formulations of some complexity. A simpler medicine would be a non-viral non-liposomal DNA formulation.

Aims

The aims of this thesis are to design, synthese and formulate an efficient and non-toxic non-viral lipopolyamine vector for possible future in-vivo application. This could be achieved by the ability of our novel polyamine (spermine) conjugates to condense DNA, leading to the formation of nanoparticles that are suitable for gene delivery.

The study will focus on the lipopolyamines synthesized from the naturally occurring tetra-amine spermine by conjugation with C18 fatty acids that vary in their degree of unsaturation to investigate and understand the effect of degree of unsaturation on the gene delivering ability of the synthesized lipopolyamine. As the first step in gene delivery is the condensation of DNA and formation of nanoparticles, this step is monitored using ethidium bromide (EthBr) fluorescence quenching assay, light scattering assay and gel electrophoresis. Also, the ability of the synthesized lipopolyamines to condense DNA will be compared with well known, in the literature, efficient DNA condensing agents and with commercially available transfection agents.

The transfection efficiency of these novel lipopolyamines will be investigated in immortalized cancer cell line (HtTA) and in a panel of primary skin cell lines (FEK4, FCP4, FCP5, FCP7 and FCP8) that are known for their resistance to accept foreign genes. Also, the cytotoxicity of these compounds will be investigated in the same cell lines used in the transfection experiments.

Finally, characterization of the lipoplex nanoparticles will be investigated by measuring the particle size of the lipoplexes to understand the role of the lipoplex nanoparticle in improving the transfection efficiency. Also, zeta potential of the lipoplexes formed will be investigated to investigate the stability of the nanoparticle.

CHAPTER 2

Materials and Methods

Chemicals

Polyamines (spermine, PEI and PLL), ethyl trifluoroacetate, 1,3-dicyclohexylcarbodiimide (DCC), *N*-hydroxy-benzotriazole (HOBt), oleic acid, oleoyl chloride, stearoyl chloride, linoleoyl chloride, triethylamine, ethidium bromide (EthBr), NaCl, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), ethylenediaminetetraacetic (EDTA) sodium salt, tris-acetate-EDTA (TAE 1x), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), ninhydrine, were routinely purchased from Sigma-Aldrich unless otherwise stated and used without purification.

Analytical thin layer chromatography (TLC) was conducted on pre-coated silica gel plates (Merck TLC aluminium sheets silica 60 F_{254}) from Merck (VWR International Ltd). Chromatograms were visualised with UV light (254 or 366 nm) or stained with ninhydrin (ninhydrin solution (0.5 % w/v in 97 % n-butanol and 3 % acetic acid) was used for spot detection. Flash column silica gel was obtained from BDH or Merck (VWR International Ltd). Preparative flash column chromatography was performed on columns packed with slurry of silica gel 60 (30-75 μm) suspended in the solvent system used in the separation process, either under gravity or under pressure with an aquarium–type pump. Sand (acid washed) used in the preparation of silica gel column was from Sigma-Aldrich. Sand was pre-washed with the solvent used in each separation system.

All organic solvents either GPR or HPLC grade were purchased from Fisher. Solvents were evaporated using a rotary evaporator (Buchi R411) in a temperature controlled water bath (Buchi B480) under a vacuum system PC2001 Vario™ (BrandTech Scientific). *Luria Bertani* (LB) broth, LB agar and ampicillin were purchased from Sigma-Aldrich. *Escherichia coli* competent cells JM109, high efficiency (>10^8 cfu/μg) were purchased from Promega.

LB broth and LB agar were prepared by dissolving the specified amount of powder in 1 L milliQ water according to the formula shown below. Both LB agar

and LB broth were autoclaved (121 °C, 15 lb/inch^2) for 30 mins, then both media were allowed to cool to 40 °C.

LB broth is composed of:

Tryptone (pancreatic digest of casein)	2 parts (10 g/L)*
Yeast extract	1 part (5 g/L)
NaCl	1 part (5 g/L)

*(g/L) is the concentration of each ingredient after preparation of 20 g/L milliQ water as recommended by the manufacturer.

LB agar is composed of:

Agar	3 parts (15 g/L)*
Tryptone (pancreatic digest of casein)	2 parts (10 g/L)
Yeast extract	1 part (5 g/L)
NaCl	1 part (5 g/L)

*(g/L) is the concentration of each ingredient after preparation of 35 g/L milliQ water as recommended by the manufacturer.

Cell culture chemicals used for media preparation were purchased from Gibco used without further purification. Lipofectin and Lipofectamine were purchased from Invitrogen, Transfectam® was from Promega and Foetal calf serum FCS was from PAA laboratories. Autoclaved (sterile) Phosphate Buffered Saline (PBS) pH 7.3 contains, 0.01 M phosphate buffer, 0.0027 M KCl and 0.137 M NaCl in MilliQ water. Trypsin was diluted to a working concentration at 0.25 % w/v with PBS.

pEGFP vector (4.7 kbp) was purchased from Clontech, pSV-β-galactosidase (pβ-gal) vector (6.8 kbp) obtained from Promega, and deoxyribonucleic acid from calf thymus, Activated and lyophilized powder purchased from Sigma-Aldrich. Deoxyribonucleic acid, from herring sperm purchased from Sigma-Aldrich. Plasmids were isolated and purified using HiSpeed™ plasmid Maxi kit, obtained from Qiagen. MilliQ water was obtained from a MilliQ PF plus system with ultra-filtration cartridge, Millipore Ltd., free of DNase and RNase.

Suppliers

Chemicals, reagents, kits, solvents, plasmids, disposables, instruments were obtained from UK suppliers (unless otherwise stated):

1. **BDH**, VWR International Ltd, Merck House, Poole, Dorset.
2. **Beckman Coulter**, Oakley Court, Kingsmead Business Park, London Road, High Wycombe, Buckinghamshire.
3. **Becton Dickinson Biosciences**, 21 Between Towns Road, Cowley, Oxford.
4. **Biochrom Ltd**, Cambridge Science Park, Milton Road, Cambridge.
5. **Bio-Rad Laboratories Ltd.**, Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire.
6. **BrandTech Scientific**,11 Bokum Rd, Essex.
7. **Buchi**, Buchi UK Ltd, 5 Whitegate Business Centre, Jardine Way, (off) Broadway, Chadderton, Oldham.
8. **Clontech**, Clontech BD Biosciences, Between Towns Road, Cowley Oxford.
9. **Corning Ltd.**, Fisher Scientific UK Ltd., Bishop Meadow Road, Loughborough, Leicestershire.
10. **Eppendorf**, Fisher Scientific UK Ltd., Bishop Meadow Road, Loughborough, Leicestershire.
11. **Falcon**, Fisher Scientific UK Ltd., Bishop Meadow Road, Loughborough, Leicestershire.
12. **Fisher**, Fisher Scientific UK Ltd., Bishop Meadow Road, Loughborough, Leicestershire.
13. **Gibco**, Gibco-Invitrogen Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley.
14. **Grant**, Keison Products, Chelmsford, Essex.
15. **Heraeus**, Kendro Laboratory Products Plc, Stortford Hall Park, Bishop's Stortford, Hertfordshire.
16. **Intermed MDH Ltd.**, Kenn Road, Clevedon, Somerset.
17. **Invitrogen Ltd**, 3 Fountain Drive, Inchinnan Business Park, Paisley.
18. **Kodak**, Hemel Hempstead, Hertfordshire.
19. **Malvern Instruments Ltd**, Enigma Business Park, Grovewood Road, Malvern, Worcestershire.

20. **Merck**, VWR International Ltd., Merck House, Poole, Dorset.
21. **Millipore UK Ltd.**, Units 3&5 the Courtyards, Hatters Lane, Watford.
22. **New Brunswick Scientific**, 17 Alban Park, Hatfield Road, St. Albans, Hertfordshire.
23. **NUNC**, Fisher Scientific UK Ltd Bishop Meadow Road, Loughborough, Leicestershire.
24. **PAA laboratories Ltd,** 1 Technine, Guard Avenue, Houndstone Business Park, Yeovil, Somerset.
25. **Promega**, Delta House, Southampton Science Park, Southampton.
26. **Qiagen Ltd.**, Qiagen House, Fleming Way, Crawley, West Sussex.
27. **Rank Brothers Ltd.**, 56 High Street, Bottisham, Cambridge
28. **Sanyo**, Integrated Services TCP Inc., Palisades Park, New Jersey, USA.
29. **Sigma-Aldrich Company Ltd**. The Old Brickyard, New Road, Gillingham, Dorset.
30. **Syngene**, Beacon House, Nuffield Rd., Cambridge.
31. **Wilovert Hund,** the Helmut Hund Ltd. Company, Helmut Hund GmbH, Wilhelm-Will-Str. 7, Wetzlar, Germany.

Methods

Amplification and purification of plasmid DNA

pEGFP and pβ-gal plasmids

DNA plasmid encoding enhanced green fluorescent protein (EGFP) under CMV promoter, obtained from Clontech, was used as reporter gene in this study. The pSV-β-galactosidase control vector (pβ-gal) with SV40 early promoter and enhancer drive transcription of the *lac*Z gene, which encodes the β-galactosidase enzyme, from Promega, was used in DNA condensation studies. DNA can amplifie on a small scale (less than 20 μg yield) using Minipreps Kit from Promega or in a large scale (up to 750 μg) using Qiagen HiSpeed™ plasmid Maxi kit (Qiagen Ltd.). Large-scale preparation of plasmids DNA protocol, described below, is based on a modified method described by Sambrook et al. (190) and using Qiagen anion-exchange resin.

A positively charged diethylaminoethanol (DEAE) resin was used to separate negatively charged phosphate groups of DNA, by properly adjusting the NaCl concentration of the elution solution. 1.2-1.6 M Aqueous NaCl solution was used to elute DNA and separate the plasmid.

Plasmids amplification

Both plasmids, pEGFP and pβ-gal, were transformed into *E. coli* JM 109 bacterial host strain. The transformed cells were grown in larger quantities (0.2–0.3 L) of LB broth supplemented with 100 mg/L ampicillin as follows:

LB broth was prepared by dissolving LB broth base powder (10 g) in 500 ml milliQ water. LB agar, for the preparation of agar plates, was prepared by dissolving LB agar powder (17.5 g) in 500 ml milliQ water. Both LB agar and LB broth were autoclaved (121 °C, 15 lb/inch^2) for 30 mins. Then, both media were left to cool down to 40 °C. Ampicillin solution (0.1 g/ml in milliQ water, 500 µl) was added to both (500 ml) media and mixed well.

Sterile LB broth can be kept at 20 °C in a tightly closed bottle for one month. To prepare LB agar plates, about 10 ml of warm LB agar was poured in a 9 cm plate, obtained from Fisher, using aseptic techniques. LB agar was then left for 30 mins to cool. LB agar plates were then incubated for 20 mins in a hot room (37 °C) upside down until dry. The solidified agar plates were kept in a cold room (4 °C) for use within 3 months.

Transformation of competent cells and plasmid propagation

Heat shock technique was used for *E. coli* JM 109 transformation. Sterile Falcon polypropylene tubes (50 ml) obtained from Falcon were chilled on ice one per transformation. Competent cells (*E. coli* JM 109) were thawed from -80 °C on ice. The thawed competent cells were gently mixed by flicking, and then 100 µl were transferred to each chilled tube. For each 100 µl competent cells, 50-100 ng of DNA was added and mixed by flicking. Tubes were placed on ice for 10 mins. Cells were

heat-shocked for 45-50 seconds in a temperature controlled water bath (Grant) at 42 °C without shaking. Tubes then immediately put on ice for 2 mins. 900 µl of cold (4 °C) LB broth was added and the tubes were incubated for 60 mins at 37 °C with shaking (200 rpm) using a temperature-controlled flask shaker (New Brunswick Scientific).

For each transformation, 100 µl transformed cells were plated on LB agar medium containing 100 µg/ml ampicillin. Plates were then incubated upside down for 18 h in an incubator (Heraeus Instruments) at 37 °C. A single colony was isolated from LB plate and incubated in 2 ml of LB broth containing ampicillin (100 µg/ml) in a 50 ml Falcon tube. The culture was grown for 8 h at 37 °C with shaking (200 rpm). The culture was then scaled up by adding the starter culture (2 ml) into a flask (at least one litre size flask) containing LB broth (250 ml) with ampicillin (100 µg/ml). The flask was then incubated for 18 h at 37 °C with shaking (200 rpm) using a temperature-controlled flask shaker.

Long term storage of transformed cells

A single colony was isolated from LB plate and incubated in 1-2 ml of LB broth containing ampicillin. The culture was grown for 8 hrs. Glycerol solution (30 % v/v) was prepared and sterile filtered. The culture (0.5 ml) was mixed with 0.5 ml of sterile glycerol solution and transferred to a cryo-preservation vial, obtained from Corning Ltd., then stored at -80 °C.

Plasmids isolation and purification

After incubating the 250 ml bacterial culture flask for 18 h at 37 °C with shaking (200 rpm) using a temperature-controlled flask shaker, cells were harvested by centrifugation, using Beckman floor centrifuge model J2-MC (Beckman Coulter) at 6000 rpm using Beckman JA-10 rotor for 15 mins at 4 °C. Cell pellets, obtained after completely removing the supernatant liquid, were resuspended in 10 ml ice-cooled P1 buffer (50 mM Tris-Cl, 10 mM EDTA and 100 µg/ml RNase A, pH 8.0), complete mixing was allowed by vortexing until no cell clumps remain. Then, 10 ml

of P2 lysis buffer (200 mM NaOH, 1 % w/v sodium dodecyl sulfate) was added to the cell suspension. The tube was then inverted gently 4 times and incubated at 20 °C for 5 mins. Ice-cooled P3 neutralisation buffer (10 ml, 3.0 M potassium acetate, pH 5.5) was added into the cell lysates and the mixture was inverted gently 4 times. The lysate was poured into the barrel of the Qiafilter cartridge and incubated for 10 mins, with the outlet nozzle closed.

The HiSpeed Maxi Tip (a syringe column with a filtration unit) was equilibrated with 10 ml buffer QBT (750 mM NaCl, 50 mM 3-[*N*-morpholino] propanesulfonic acid (MOPS), 15 % v/v isopropanol, 0.15 % v/v Triton X-100) and the column was allowed to empty by gravity flow. The outlet nozzle cap of the Qiafilter cartridge was removed and the plunger was gently inserted into the cartridge. The filtrate was collected into the equilibrated Maxi Tip and allowed to be filtered through the resin. After that, 60 ml of buffer QC (1.0 M NaCl, 50 mM MOPS, 15 % v/v isopropanol, pH 7.0) was added to wash the HiSpeed Maxi Tip. Entrapped DNA in the resin was eluted by adding 15 ml of buffer QF (1.25 M NaCl, 50 mM Tris-Cl, 15 % v/v isopropanol, pH 8.5) into the tip. The eluted solution was kept in a 50 ml Falcon tube. Isopropanol (10.5 ml) was then added and mixed well to precipitate the DNA, then the mixture was incubated for 5 mins at 20 °C.

A DNA collection unit (QIA precipitator tip) was attached to the nozzle of a 30 ml syringe from which a plunger was removed. Then, the eluate/isopropanol mixture that has the precipitated DNA was added to the precipitator unit, and the plunger was inserted into the syringe using constant pressure. DNA was entrapped in the precipitator unit. Then, the tip and the plunger were removed from the syringe. After that, the tip was reconnected again to the syringe nozzle and 2 ml of 70 % v/v EtOH was added into the syringe. DNA was washed by pressing EtOH through the QIA precipitator using inserted plunger at a constant pressure. The tip and the plunger were removed from the syringe and DNA was dried by pushing air through the precipitator 2-3 times and the outlet nozzle of the precipitator was dried with absorbent paper.

To elute the DNA in the tip, 1 ml of buffer TE (10mM Tris-HCl, 1mM EDTA, pH 8) was added into a new 5 ml syringe to which the precipitator tip was connected.

The plunger was attached and pushed gently. The eluted solution was collected in an ultracentrifuge tube (1.5 ml), obtained from Eppendorf Ltd. The previous step was repeated by the transfer of the eluate in the 1.5 ml ultracentrifuge tube to the 5 ml syringe to which the precipitator tip was connected and eluted again in the same 1.5 ml tube. DNA solution was stored at -20 °C.

Analysis of the purification procedure

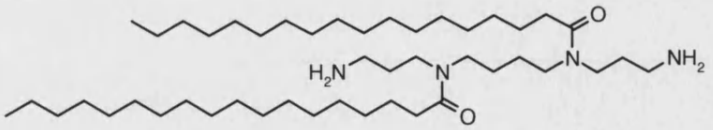
The DNA obtained can be quantified by the maximum UV absorption at 260 nm. This wavelength is an average of the absorption of the individual nucleotides which vary between 256 – 281 nm. DNA was determined by measuring the maximum absorption at 260 nm for nucleic acids and 280 nm for proteins. Ratios of A_{260}/A_{280} between 1.75–1.90 are considered acceptable for DNA purity (191, 192).

Plasmid DNA yields and quality were analyzed spectrophotometrically using Gene Quant II RNA/DNA calculator (Biochrom Ltd). Plasmid DNA sample 5 µl was diluted to 100 µl with TE buffer, in a microcuvette. The absorbance was measured at 260 nm (A_{260}) and 280 nm (A_{280}). TE buffer 100 µl was used as a standard reference. The calculation for DNA concentration by dilution factor 20, and a conversion factor for double stranded dsDNA, 50 µg/ml/A_{260}.

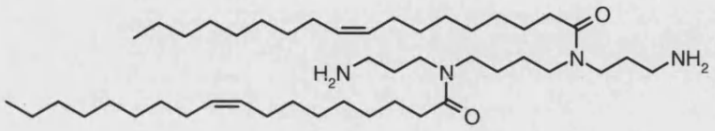
Lipopolyamines synthesis

Spermine was used as the starting material for the synthetic process that was protected on both the primary amino functional groups. Then acylation of the protected spermine was carried out, to covalently bound two fatty acyl chains on the free secondary amines of the protected spermine, either by the fatty acid or fatty acyl chloride followed by deprotection to afford our novel lipospermines (Fig. 1) used in this study.

N^4,N^9-Distearoyl spermine



N^4,N^9-Dioleoyl spermine



N^4,N^9-Dilinoleoyl spermine

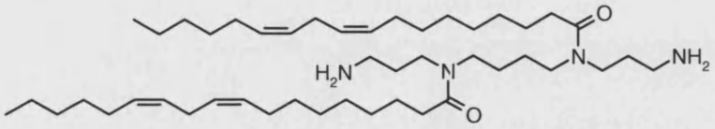


Figure 1. Our novel lipospermines, N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine and N^4,N^9-dilinoleoyl spermine investigated in this study.

Synthesis of N^4,N^9-dioleoyl spermine

Synthesis of N^1,N^{12}-ditrifluoroacetyl spermine

Spermine 1g (4.94 mmole) was dissolved in methanol (MeOH, 20 ml). A primary amino protecting group, ethyl trifluoroacetate (2.2 eq) was added drop wise. The reaction mixture was then stirred using a magnetic stirrer (Büchi, Büchi Labortechnik) for 18 h at 25 °C. The solvent was evaporated to dryness in vacuo to form N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane which was homogenous on silica gel thin-layer chromatography (MeOH-conc. aq. NH_3 4:1 v/v) with $R_f = 0.8$ compared to the starting substance spermine $R_f = 0.1$ using the same solvent system. Ninhydrin solution (0.5 % w/v in 97 % n-butanol and 3 % acetic acid) was used for spot detection.

Synthesis of N^1,N^{12}-ditrifluoroacetyl-N^4,N^9-dioleoyl spermine

DCC 2.5 eq, HOBt 0.2 eq and oleic acid (2.2 eq) were added to the diprotected spermine solution in dichloromethane CH_2Cl_2 and MeOH (1:1 v/v, 15 ml). The reaction solution was stirred for 18 h at 25 °C. The solvent was evaporated to dryness in vacuo. The tetra-amide residue was re-dissolved in CH_2Cl_2 for DCU to precipitate. The solution was then filtered to give pale yellow filtrate that was evaporated to dryness in vacuo to form N^4,N^9-dioleoyl-N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane. The tetra-amide was detected on silica gel thin-layer chromatography by the disappearance of the diprotected spermine (N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane) spot and compared to the starting substance spermine $R_f = 0.1$ and oleic acid $R_f = 0.2$. The TLC plate was observed under UV for C=C double bond of oleic acid, and then derivatised with ninhydrin solution (0.5 % w/v in 97 % n-butanol and 3 % acetic acid) was used for spot detection.

Deprotection of N^1,N^{12}-ditrifluoroacetyl-N^4,N^9-dioleoyl spermine

For the removal of the di-trifluoroacetyl groups, the tetra-amide was dissolved in methanol and the pH of the solution was increased by saturating with ammonia gas, then it was left for 18 h at 25 °C with stirring. The solution was then evaporated to dryness in vacuo, to remove ammonia and all solvents, to give a residue which was purified by column chromatographic elution over flash silica gel using two mobile phase systems. First system, CH_2Cl_2-MeOH 5:3 v/v, to remove unprotonated compounds in the reaction mixture (e.g. oleic acid and remaining DCU). This followed by elution with CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v to afford N^4,N^9-dioleoyl spermine that was homogenous on silica gel thin-layer chromatography CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v $R_f = 0.44$. The lipopolyamine was characterized by low and high-resolution mass spectroscopy (HRMS) measured on a VG Autospec Q spectrometer.

Synthesis of N^4,N^9-distearoyl spermine

Synthesis of N^1,N^{12}-ditrifluoroacetyl-N^4,N^9-distearoyl spermine

Triethylamine 2.5 eq, stearoyl chloride 2.2 eq were added to the diprotected spermine solution in CH_2Cl_2 and MeOH (1:1 v/v, 15 ml). The reaction solution was stirred for 72 h at 20 °C. The solvent was evaporated to dryness in vacuo to form N^4,N^9-distearoyl-N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane. The tetra-amide spermine was detected on silica gel thin-layer chromatography by the disappearance of the diprotected spermine (N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane) spot and compared to the starting material spermine $R_f = 0.1$ and oleic acid $R_f = 0.2$.

Deprotection of N^1,N^{12}-ditrifluoroacetyl-N^4,N^9- distearoyl spermine

For the removal of the di-trifluoroacetyl groups, the tetra-amide was dissolved in methanol and the pH of the solution was increased by saturating with ammonia gas, then it was left for 18 h at 25 °C with stirring. The solution was then evaporated to dryness in vacuo, to give a residue which was purified by column chromatographic elution over flash silica gel using two mobile phase systems. CH_2Cl_2-MeOH 5:3 v/v, followed by elution with CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v to afford N^4,N^9-distearoyl spermine that was homogenous on silica gel thin-layer chromatography CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v $R_f = 0.41$ and was characterized by HRMS.

Synthesis of N^4,N^9-dilinoleoyl spermine

Synthesis of N^1,N^{12}-ditrifluoroacetyl-N^4,N^9-dilinoleoyl spermine

Triethylamine 2.5 eq, linoleoyl chloride 2.2 eq were added to the diprotected spermine solution in pyridine. The reaction solution was stirred for 72 h at 20 °C. The solvent was evaporated to dryness in vacuo to form N^4,N^9-dilinoleoyl-N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane. The tetra-amide spermine was detected on silica gel thin-layer chromatography by the disappearance of the

diprotected spermine (N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane) spot and compared to the (original) starting materials spermine $R_f = 0.1$ and oleic acid $R_f = 0.2$. The TLC plate was observed under UV for C=C double bond of linoleic acid, and then derivatised with ninhydrin solution.

Deprotection of N^1,N^{12}-ditrifluoroacetyl-N^4,N^9-dilinoleoyl spermine

For the removal of the di-trifluoroacetyl groups, the tetra-amide was dissolved in methanol and the pH of the solution was increased by saturating with ammonia gas, then it was left for 18 h at 25 °C with stirring. The solution was then evaporated to dryness in vacuo, to give a residue which was purified over silica gel CH_2Cl_2-MeOH 5:3 v/v, followed by CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v to afford N^4,N^9-dilinoleoyl spermine that was homogenous on silica gel thin-layer chromatography CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v $R_f = 0.49$ and was characterized by HRMS.

DNA condensation studies

DNA condensation was carried out to investigate the neutralization of the negative charges of the phosphate groups on the DNA back bone. Neutralization of the negative charges leads to collapse of DNA into a more compact structure (nanoparticles). Nanoparticles formation improves the delivery across cellular barriers (as described before) and hence increases the transfection efficiency of the delivered DNA. These studies include:

1. Ethidium bromide fluorescence quenching assay
2. Light scattering assay
3. Gel electrophoresis study

Ethidium bromide fluorescence quenching assay

Each concentration of the DNA (calf thymus DNA, herring testes, pEGFP and pβ-gal) stock solutions (approximately 1 μg/μl) was determined spectroscopically and 6 μg (approximately 6 μl) of DNA was diluted to 3 ml with HEPES buffer (20 mM

NaCl, 2 mM HEPES,10 μM EDTA, pH 7.4) in a glass cuvette stirred with a micro-flea. Immediately prior to analysis, EthBr solution (3 μl, 0.5 mg/ml) was added to the stirring solution and allowed to equilibrate for 10 mins.

Aliquots (5 μl) of spermine (10 x 0.04 mg/ml, and then 5 x 0.2 mg/ml) were then added to the stirring solution and the fluorescence measured after 1 min equilibration using Perkin-Elmer LS 50B luminescent spectrometer (λ_{excit} = 260 nm and λ_{emiss} = 600 nm with slit width 5 nm) while stirring using an electronic stirrer (Rank Brothers Ltd.). The total polyamine solution added to the DNA solution did not exceed 5 % of the total volume of the solution, so no correction was made for sample dilution. The fluorescence was expressed as the percentage of the maximum fluorescence when EthBr was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free EthBr in solution (193). This correction and the subsequent normalization were performed using an Excel spreadsheet.

In the case of poly-L-lysine (PLL, average molecular weight. 9,600 Da (PLL 9.6k) and 27000 Da (PLL 27k) the same amounts of DNA were used and aliquots of PLL 9.6k were (5 μl) of (10 x 0.08 mg/ml, and then 6 x 0.2 mg/ml) were used. For PLL 27k, aliquots of (5 μl) of (8 x 0.16 mg/ml) were used. Aliquots were then added to the stirring solution of 3 ml HEPES buffer in a glass cuvette that have 6 μg DNA and 1.5 μg EthBr. The fluorescence was measured after 1 min equilibration using Perkin-Elmer LS 50B luminescent spectrometer (λ_{excit} = 260 nm and λ_{emiss} = 600 nm with slit width 5 nm) while stirring using an electronic stirrer (Rank Brothers Ltd.). The total polyamine solution added to the DNA solution did not exceed 5 % of the total volume of the solution, so no correction was made for sample dilution. The fluorescence was expressed as the percentage of the maximum fluorescence when EthBr was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free EthBr in solution. This correction and the subsequent normalization were performed using an Excel spreadsheet.

In the case of polyethylenimine (PEI average molecular weight 2000 Da (PEI 2k) and 60000 Da (PEI 60k)) the same amounts of DNA were used and aliquots of

PEI 2k were (5 µl) of (10 x 0.3 mg/ml) were used. For PEI 60k, aliquots of (5 µl) of (10 x 0.3 mg/ml) were used. Aliquots were then added to the stirring solution of 3 ml HEPES buffer in a glass cuvette that have 6 µg DNA and 1.5 µg EthBr. The fluorescence was measured after 1 min equilibration using Perkin-Elmer LS 50B luminescent spectrometer (λ_{excit} = 260 nm and λ_{emiss} = 600 nm with slit width 5 nm) while stirring using an electronic stirrer (Rank Brothers Ltd.). The total polyamine solution added to the DNA solution did not exceed 5 % of the total volume of the solution, so no correction was made for sample dilution. The fluorescence was expressed as the percentage of the maximum fluorescence when EthBr was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free EthBr in solution. This correction and the subsequent normalization were performed using an Excel spreadsheet.

In the case of the synthesized lipopolyamines, N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine and N^4,N^9-dilinoleoyl spermine, the lipopolyamines were prepared as stock solutions using absolute EtOH as solvent and diluted with HEPES buffer to the specified concentrations directly before use. Aliquots were added to the stirring solution of 3 ml HEPES buffer in a glass cuvette that have 6 µg DNA and 1.5 µg EthBr. The fluorescence was measured after 1 min equilibration using Perkin-Elmer LS 50B luminescent spectrometer (λ_{excit} = 260 nm and λ_{emiss} = 600 nm with slit width 5 nm) while stirring using an electronic stirrer (Rank Brothers Ltd.). The total lipopolyamine solution added to the DNA solution did not exceed 5 % of the total volume of the solution, so no correction was made for sample dilution. The fluorescence was expressed as the percentage of the maximum fluorescence when EthBr was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free EthBr in solution. This correction and the subsequent normalization were performed using an Excel spreadsheet.

In the case of the commercially available transfecting agents, Lipofectin, Lipofectamine (available as liposomal formulations) and the non-liposomal formulation Transfectam®, aliquots were added to the stirring solution of 3 ml HEPES buffer in a glass cuvette that have 6 µg DNA and 1.5 µg EthBr. The fluorescence was measured after 1 min equilibration using Perkin-Elmer LS 50B

luminescent spectrometer (λ_{excit} = 260 nm and λ_{emiss} = 600 nm with slit width 5 nm) while stirring using an electronic stirrer (Rank Brothers Ltd.). The total lipopolyamine solution added to the DNA solution did not exceed 5 % of the total volume of the solution, so no correction was made for sample dilution. The fluorescence was expressed as the percentage of the maximum fluorescence when EthBr was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free EthBr in solution. This correction and the subsequent normalization were performed using an Excel spreadsheet.

The DNA polyamine complex composition is expressed as N/P (ammonium/phosphate) as follows:

$$\text{N/P} = \frac{\textit{Ammonium equivalents of the cationic compound}}{\textit{Phosphate equivalents of the DNA used}} \qquad [1]$$

Ammonium equivalents of the polyamine were determined from pK_a value of each amino group at pH 7.4 (HEPES buffer) according to Henderson-Hasselbach equation:

$$\text{pH} = \text{p}K_a + \log_{10} \frac{[\textit{conjugate base}]}{[\textit{conjugate acid}]} \qquad [2]$$

The charge ratio was calculated according to the ammonium/phosphate (+/-) charge ratio of the investigated lipopolyamines to DNA. For DNA it was calculated as 330 g/mole is DNA that contains one negative charge (5). In the case of N^4,N^9-distearoyl spermine it was calculated as 735.7g/mole that contains two primary amino groups that are protonated (positively charged) at pH 7.4. In the case of N^4,N^9-dioleoyl spermine it was calculated as 731.26 g/mole that contains two primary amino groups that are protonated (positively charged) at pH 7.4. In the case of N^4,N^9-dilinoleoyl spermine it was calculated as 727.7g/mole that contains two primary amino groups that are protonated (positively charged) at pH 7.4.

In the case of the commercially available lipopolyamine (DOGS, Transfectam®) it was calculated as 1263 g/mole that contains three nitrogens (more

accurately 2.92) that are protonated at pH 7.4, although Transfectam® has four nitrogens (two secondary amines and two primary amines) that can be protonated in strong acid.

Charge ratios of PEI 2k and PEI 60k were calculated by multiplying the weight ratio of PEI to DNA by 1.9 (75). In the liposomal formulation Lipofectin, the cationic lipid DOTMA carries 1 positive charge (ammonium eq/mole) (16) while the cationic lipid DOSPA in the Lipofectamine formulation carries 4 positive charges (ammonium eq/mole) (185). In the non-liposomal formulations, Transfectam® carries 3 positive charges (185), while N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine and N^4,N^9-dilinoleoyl spermine each lipopolyamine carries 2 positive charges.

Binding constant calculation

From EthBr fluorescence quenching results for DNA interaction with the condensing agent, the binding constant of the DNA condensing agent (vector) to DNA can be calculated and compared by the loss of EthBr fluorescence as a function of DNA condensing agent concentration. The drug concentration producing 50 % inhibition of fluorescence is approximately inversely proportional to the binding constant (194). Denny and co-workers (195) established the binding equations of DNA to both EthBr and vectors as follows:

$$[DNA]+[vector]\leftrightarrow[DNA\text{–}vector] \qquad [3]$$

$$[DNA]+[EthBr]\leftrightarrow[DNA\text{–}EthBr] \qquad [4]$$

$$K_b\ (\text{vector}) = \frac{[DNA\text{–}vector]}{[DNA]\times[vector]} \qquad [5]$$

$$K_b\ (\text{EthBr}) = \frac{[DNA\text{–}EthBr]}{[DNA]\times[EthBr]} \qquad [6]$$

$$K_b\ (\text{vector}) = \frac{[DNA\text{–}vector]\times[EthBr]\times K_b\,(EthBr)}{[vector]\times[DNA-EthBr]} \qquad [7]$$

At 50 % residual fluorescence, the ratio of [DNA-vector] over [DNA-EthBr] equal to 1. So, the "apparent" equilibrium constant (K_b) of polyamine binding was calculated, taking into account that Morgan et al. (194) reported that the binding constant of ethidium bromide was 10^7 M^{-1}, as follows:

$$K_b \text{ (vector)} = \frac{[EthBr]\times 10^7}{[vector]} \qquad [8]$$

The concentration of the condensing agent producing 50 % intercalated EthBr fluorescence inhibition was calculated experimentally.

Light scattering assay

A light scattering experiment was designed to investigate particle formation as a result of DNA condensation by polyamines and lipopolyamines. The apparent UV absorbance at 320 nm, where there is no DNA absorbance interference above 300 nm was measured showing light scattering (i.e. decreased light transmission) (145).

DNA (linear calf thymus or herring testes) was purchased from Sigma-Aldrich. Plasmids DNAs pEGFP and pβ-gal were prepared by MaxiPrep as mentioned before. All DNA solutions were diluted using TE buffer and their concentrations were measured by GeneQuant II spectrophotometer. HEPES buffer was made of 2 mM HEPES, 20 mM NaCl, 10 μM EDTA, and MilliQ water, the pH was adjusted to 7.4 with aqueous NaOH solution. HEPES buffer was filtered through a 0.22 μm membrane prior to use.

Spermine (17 mg) was dissolved in MilliQ water (1 ml as a stock solution). DNA 60 μg, of 1 mg/ml solution) diluted to 3 ml with HEPES buffer in a cuvette with a micro-flea, and the concentration determined spectroscopically (Milton Roy Spectronic 601 spectrometer, 1 cm path length, 3 ml cuvette). UV measurement of DNA in buffer was performed and adjusted to zero. Then, aliquots (5 μl) of spermine (10 x 0.4 mg/ml, and then 5 x 2.0 mg/ml) were then added to the stirring

solution and the absorbance (light scattering) at 320 nm measured after 1 min stirring to allow the mixture to reach equilibrium. The Apparent UV absorbance values were then plotted against N/P ratios.

In the case of poly-L-lysine (PLL 9.6k and PLL 27k) the same amounts of DNA were used and aliquots of PLL 9.6k were (5 µl) of (10 x 0.8 mg/ml, and then 6 x 2.0 mg/ml) were used. For PLL 27k, aliquots of (5 µl) of (8 x 1.6 mg/ml) were used. In the case of polyethylenimine (PEI 2k and PEI 60k) the same amounts of DNA were used and aliquots of PEI2k were (5 µl) of (10 x 3.0 mg/ml) were used. For PEI 60k, aliquots of (5 µl) of (10 x 3.0 mg/ml) were used. In the case of the synthesized lipopolyamines, N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine and N^4,N^9-dilinoleoyl spermine, the lipopolyamines were prepared as stock solutions using absolute EtOH as solvent and diluted with HEPES buffer to the specified concentrations directly before use.

Gel electrophoresis study

1 gm of agarose (electrophoresis grade, Gibco) was added to 100 ml of 1x TAE (Tris-Acetate-EDTA, 40 mM Tris-Acetate and 1 mM EDTA) buffer. The mixture was then heated in a microwave oven to boiling for 2 mins with swirling the flask 3-4 times while boiling to dissolve the agarose. When the agarose is completely dissolved, the solution was then allowed to cool to a consistency that can be poured. Then EthBr was added to a concentration of 1.0 µg/ml. When the gel is cooling down, the gel tray was securely sealed at the ends with tape strips to form a fluid-tight seal. The comb was placed over the gel tray. After that, when agarose has cooled to about 60°C, the agarose was poured into the gel tray to a depth of 4-8 mm. Then the gel was allowed to set at room temperature for 30 mins followed by refrigeration for further 15 mins for complete solidification of the gel. The comb was removed from the solidified gel as well as the tape from the edges of the gel tray. The gel was then transferred to the electrophoresis chamber, Kodak Biomax QS 710 (Kodak), and submerged into the electrophoresis buffer (TAE buffer).

For pEGFP lipoplex formation, two solutions were prepared for each well, solution A (DNA) and solution B (lipopolyamine). Solution A was prepared by diluting DNA solution (0.5-1 μg/well) to 5 μl using 1x TAE buffer, in a sterile 1.5 ml micro-centrifuge tube obtained from Eppendorf Ltd., gently mixed using a vortex, for 2 secs and incubated for 30 mins at room temperature. Solution B was prepared by diluting the lipopolyamine solution to 10 μl using 1x TAE buffer, in a sterile 1.5 ml micro-centrifuge tube, gently mixed using a vortex, for 2 secs and incubated for 30 mins at room temperature. The complex was then prepared by mixing solution A and B (vortex) for 1 sec then incubated for 20 mins at room temperature.

Each lipopolyamine used in solution B was at a different volume according to the required N/P ratio for 0.5-1 μg DNA/well, as described before in the N/P determination section for EthBr assay. In the non-liposomal formulations, Transfectam®, DOGS carries 3 positive charges (185), while N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine and N^4,N^9-dilinoleoyl spermine each of these lipopolyamines carries 2 positive charges (on their two primary amines).

Samples (15 μl) of plasmid DNA (0.5 μg), either free or complexed with the lipopolyamine (according to the charge ratio) were mixed with the loading dye (blue/orange loading dye 6X, Promega) and loaded into the wells. The loading dye is used at a 1X final concentration and contains 0.03 % xylene cyanol FF (4 kbp migration), 0.03 % bromophenol blue (300 bp migration), 0.4 % orange G (50 bp migration), 10mM Tris-HCl (pH 7.5) and 50mM EDTA (pH 8). Electrophoresis at 75 V/cm (Bio-Rad Power Pack 300, Bio-Rad Laboratories Ltd) was carried out for 1 hour. The gel was removed and the (unbound) free DNA in the agarose gel was visualized under UV using GeneGenius (Syngene).

Tissue culture and transfection experiments

Primary skin cell lines

Six primary cell lines were used in the transfection experiments, FEK4 (196, 197), FCP4, FCP5, FCP7 and FCP8 cells are human primary fibroblasts derived from

newborn foreskin explants (198), which were used as a model for human primary cells (passage dependent cell line). FEK4 cells were kindly provided by Prof. R. M. Tyrrell, Department of Pharmacy and Pharmacology, University of Bath, UK. Cells were cultured in Earle's Minimal Essential Medium (EMEM) with a final Foetal calf serum (FCS) concentration of 15 % v/v according to the following formula (for 500 ml):

10X EMEM	50 ml
7.5 % $NaHCO_3$	13.5 ml
200 mM L-glutamine	5 ml
Penicillin (10,000 IU/ml)/Streptomycin (10,000 IU/ml)	2.5 ml
Foetal calf serum (FCS)	75 ml
Autoclaved MilliQ Water	354 ml

FCS was heat inactivated before use by defrosting at 37 °C and heating at 56 °C for 45 mins in a temperature-controlled water bath (Grant). Sterile Phosphate Buffered Saline (PBS) solution was used in all transfection experiments to wash cells and contains 0.01 M phosphate buffer, 0.0027 M KCl and 0.137 M NaCl in MilliQ water, pH 7.4. Trypsin was diluted to a working concentration at 0.25 % w/v with PBS.

Cells were visually assessed daily for evidence of microbial contamination under an inverted light microscope (Wilovert Hund). All aseptic techniques for cell culture were carried out in a laminar flow cabinet (Intermed MDH Ltd.) designed for vertical re-circulation of air. A temperature-controlled water bath (Grant) was used to warm the media for cell culture experiments. A monolayer of primary skin cell lines was grown in 25 ml EMEM supplemented with 15 % v/v FCS in 150 cm^2 (T_{150}) flasks from NUNC. Cell lines were incubated at 37 °C, 5 % CO_2, 95 % air and humidified atmosphere by using LEEC PF2 incubator (Sanyo). Primary skin cells were passaged twice, maximum once, a week and used between passages 7-15.

Primary cells were passaged (subcultured) by trypsinisation technique. The growth medium was aspirated from the flasks. Then, cells were washed twice with PBS solution (10 ml/T_{150} flask). PBS solution was then removed by aspiration. Cells

were treated with trypsin 0.25 % w/v (3 ml/T_{150} flask) and incubated for 5-10 mins at 37 °C and 5 % v/v CO_2 for trypsinisation reaction. After that, the flasks were gently tapped to assess the detachment of the cells, which were checked under microscope to ensure at least 85 % cell detachment. To stop trypsinisation, 10 ml of 15 % v/v FCS EMEM was added to the flask. The cell suspension was then transferred to a 50 ml Falcon tube, obtained from Falcon, for separation of cells by centrifugation at1000 rpm for 5 mins using a cells centrifuge, Falcon 6/300 MSE (Sanyo). Cells were re-suspended in 6 ml of 15 % v/v FCS EMEM and was seeded at a concentration of 1 x 10^6 cells/flask (T_{150}) that have 25 ml 15 % v/v FCS EMEM for next passage.

To determine the concentration of cells in a sample, a viable count was carried out using a haematocytometer slide. Stained cell suspension was prepared by mixing trypsinized cells (50 µl) with same volume of nigrosin or trypan blue (50 µl) 0.4 % (w/v) solution in PBS. The cell sample was loaded into the raised sides of the glass chambers (top and bottom) covered with a coverslip 0.1 mm above the chamber floor until the whole chamber was fully loaded. The slide was then placed under an inverted light microscope. Viable cells were detected using a bright halo light around their cell membrane. On the other hand, dead cells were permeabilized by the dye and appear as black spheres under the microscope. The viable cell number in the four squares surrounding the central square and this was repeated for the other side of the haemocytometer chamber. The viable cell concentration in the original cell suspension was calculated using the following equation:

$$Cell\ concentration\ (cells/ml) = \frac{total\ cell\ count\ in\ 4\ squares}{4} \times dilution\ factor \times 10^4 \quad [9]$$

For cell storage, cells were detached from a confluent monolayer by trypsinisation as described before followed by centrifugation at 1000 rpm form 5 mins. The supernatant was then discarded and the cell pellets were re-suspended in the growth media at a concentration of 2 x 10^5 cells/ml. A cell freezing solution (10 % v/v) of growth media with a filter-sterilized solution of dimethylsulfoxide (DMSO) as a cryopreservative was prepared. Cell suspension (0.4 ml) was dispensed with 0.6 ml of cell freezing solution into cryotubes and stored at -70 °C overnight. After that,

tubes were fitted into a Union Carbide LR-40 liquid nitrogen freezer where they were stored at approximately -150 °C (long-term, years).

For cell recovery, the contents of the cryotube were thawed rapidly by brief incubation at 37 °C water bath followed by dilution with 10 ml of fresh growth medium. Cells were centrifuged for 5 mins at 1000 rpm and the supernatant was discarded. Cell pellets were then re-suspended in 10 ml of fresh medium, and then 1 x 10^6 cells were transferred to flask (T_{150}) containing 25 ml of growth medium and incubated under standard culture conditions. Cells were cultured for at least two passages in order to establish them before any experiments were carried out.

Transfection experiments were carried out using pEGFP, encoding for green fluorescent protein as a reporter gene, either complexed with DNA condensing agents, or free (naked DNA) as a negative control. Cells were seeded in 6-well plates, obtained from NUNC. DNA was used at a concentration of 2 µg/well. DNA condensing agents used for FEK4 primary cells transfection were the liposomal formulations Lipofectin (DOTMA/DOPE 1:1 w/w), Lipofectamine (DOSPA/DOPE 3:1 w/w) and the non-liposomal formulations Transfectam® (DOGS), N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine, N^4,N^9-dilinoleoyl spermine. For FCP4, FCP5, FCP7 and FCP8 primary cells, transfection experiments were carried out using Transfectam® (DOGS), N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine and N^4,N^9-dilinoleoyl spermine.

For the DNA-vector complex formation experiment, two solutions were prepared for each well (6-well plate), solution A (DNA) and solution B (condensing agent). Solution A was prepared by diluting DNA solution (2 µg/well) to 100 µl using Opti-MEM, a serum free media (Gibco), in a sterile 1.5 ml micro-centrifuge tube obtained from Eppendorf Ltd., gently mixed using a vortex, for 2 secs and incubated for 30 mins at room temperature. Solution B was prepared by diluting transfection agents solution to 100 µl using Opti-MEM, serum free media, in a sterile 1.5 ml micro-centrifuge tube, gently mixed using a vortex, for 2 secs and incubated for 30 mins at room temperature. The complex was then prepared by mixing solution A and B with vortex for 1 sec then incubated for 20 mins at room temperature.

Each transfection agent used in solution B was at a different volume according to the required N/P ratio for 2 μg DNA/well, as described before in the N/P determination section for EthBr assay. In the liposomal formulation Lipofectin, the cationic lipid DOTMA carries 1 positive charge (ammonium eq/mole) (16) while the cationic lipid DOSPA in the Lipofectamine formulation carries 4 positive charges (ammonium eq/mole) (185). In the non-liposomal formulations, Transfectam®, DOGS carries 3 positive charges (185), while N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine, N^4,N^9-dilinoleoyl spermine each of these lipopolyamines carries 2 positive charges (on their two primary amines).

For FEK4 transfection, 1x 10^5 cells/well were seeded in 6-well plates in 4 ml of 15 % v/v FCS EMEM. Cells were incubated at 37 °C and 5 % v/v CO_2 in a CO_2 incubator for 24 h to reach 50-60 % confluency. Prior to transfection, the adherent cells were washed twice with PBS solution, and then washed once with Opti-MEM. Washed medium was removed and replaced with 800 μl of Opti-MEM to which 200 μl of the transfection complex, as a result of mixing solution A and B, was added to the plates except for control cells where 1000 μl of Opti-MEM was added (no DNA complex solution). The transfected plates were incubated for 4 h at 37 °C in an atmosphere of 5 % v/v CO_2. After that, the Opti-MEM in the incubated plates was aspirated and replaced with 2 ml of 15 % v/v FCS EMEM media and incubated for a further 44 h at 37 °C in an atmosphere of 5 % v/v CO_2.

The study using naked DNA was performed to asses if there was any transfection efficiency from naked DNA compared to the lipoplexes. The same transfection protocol used before was applied, except for solution B that contains only serum free media without any DNA condensing agent to be added. For all transfection studies, each data point represents the mean ± standard deviation (± SD) of triplicate samples and each experiment was repeated three times.

HtTA cell transfection

Human cervix carcinoma, HeLa derivative and transformed cell line (HtTA) was used as a model for carcinoma cells (an immortal cell line). The HtTA cells

being stably transfected with a tetracycline-controlled transactivator (tTA) consisting of the tet repressor fused with the activating domain of virion protein 16 of the herpes simplex virus (HSV). HtTA cells were kindly provided by Prof. R. M. Tyrrell, Department of Pharmacy and Pharmacology, University of Bath, UK. Cells were cultured in Earle's Minimal Essential Medium (EMEM) with a final FCS concentration of 10 % v/v according to the following formula (for 500 ml):

10X EMEM	50 ml
7.5 % $NaHCO_3$	13.5 ml
200 mM L-glutamine	5 ml
Penicillin (10,000 IU/ml)/Streptomycin (10,000 IU/ml)	2.5 ml
Foetal calf serum (FCS)	50 ml
Autoclaved MilliQ Water	379 ml

FCS was heat inactivated before use by defrosting at 37 °C and heating at 56 °C for 45 mins in a temperature-controlled water bath (Grant). Trypsinisation procedures were carried out using the same protocol for FEK4 sub-culturing. The culture was incubated at 37 °C in an atmosphere of 5 % v/v CO_2. HtTA cells were passaged every 3 days.

For the transfection (gene delivery) and the resultant gene activity (transfection efficiency) the same protocol for FEK4 transfection was used as described before. Briefly, HtTA cells were seeded at 1 x 10^5 cell/well in 6 well plates in 4 ml EMEM media with 10 % v/v FCS for 24 h to reach a plate confluency of 50-60 % on the day of transfection. The complex was prepared by mixing pEGFP (2 μg) with the cationic liposomes or lipopolyamine in serum free media Opti-MEM according to the charge ratio at room temperature for 30 mins, and then incubated with the cells for 4 h at 37 °C in 5 % v/v CO_2, then the cells were washed and cultured for further 44 h in growth medium at 37 °C in 5 % v/v CO_2 before the assay.

Fluorescence-activated cell sorting experiment

To analyse the transfection efficiency of the delivered pEGFP, levels of green fluorescent protein (GFP positive cells) in the transfected cells were detected using a

fluorescence activated cell sorting (FACS) machine (199) Becton Dickinson FACS Vantage dual Laser Instrument, (argon ion laser 488 nm) (Becton Dickinson Biosciences). FACS is used for to determine the fluorescent molecules (or biomolecules) inside studied cells. Individual cells pass through a laser beam allowing the light scatter and fluorescence characteristics of each cell to be measured. Filter lens (F) for emission detection was chosen based on the fluorophore used. In our experiment, EGFP is detected by F1 ($\lambda_{em} = 530 \pm 15$ nm).

Preparation of cell sample for FACS analysis

The cells were detached through aspiration of the media from 6-well plates. Then, cells were washed twice with PBS solution (2 ml/well). PBS buffer was removed by aspiration. Cells were treated with trypsin 0.25 % w/v (0.5 ml/well) and incubated for 5 mins at 37 °C and 5 % v/v CO_2 for trypsinisation reaction. After that, the plates were gently tapped to assess the detachment of the cells, which were checked under microscope to ensure at least 85 % of cell detached. To stop trypsinisation, 1 ml of 15 % v/v FCS EMEM was added for each well. The cell suspension was then transferred to FACS polystyrene test tubes (12 x 75 mm), obtained from Falcon, filled with 1 ml of 15 % FCS EMEM for separation of cells by centrifugation at1000 rpm for 5 mins at room temperature using a cells centrifuge, Falcon 6/300 MSE (Sanyo). Cells were re-suspended in PBS (1 ml/tube) for further centrifugation at1000 rpm for 5 mins at room temperature. Cell suspension for FACS analysis was obtained by dissolving the pellet in 500 µl PBS/tube. Untreated (untransfected) cell sample was used as a control in the measurement.

CELLQuest v.1.0 software (Becton Dickinson Biosciences) was used to analyse FACS data. Typically, 10,000 events were collected. Recordings were made at green fluorescence (FL1) and data were expressed as histograms. Only a subset of the data obtained from healthy cells data (major population) was analysed through a gate setting. This gating was determined from the dot plots between forward-scattered light (FSC) and side-scattered light (SSC). FSC is a parameter proportional to cell size and SSC indicates the cell granularity or internal complexity. In the histogram of events at different fluorescence intensity control group, the fluorescence intensity range (M_1) was set as a constant range throughout the experiments. For

EGFP detection, the % fluorescence cells sorting events in the established range (M_1) was reported with the correction of the background fluorescence of the control sample.

Cytotoxicity (MTT) assay for the lipoplex formed

The MTT assay is a colorimetric assay to evaluate the metabolic activity of viable cells to convert a soluble tetrazolium salt [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] into an insoluble formazan crystal. The MTT assay is used to investigate the cytotoxic effects of lipopolyamines and lipoplexes (pEGFP complexes). Six primary cell lines were used in the cytotoxicity experiments, FEK4, FCP4, FCP5, FCP7 and FCP8 cells are human primary fibroblasts derived from newborn foreskin explants, which were used as a model for human primary cells (mortal cell line), and these cells were cultured in Earle's Minimal Essential Medium (EMEM) with a final serum concentration of 15 % v/v. HtTA was used as a model for carcinoma cells (an immortal cell line), and these HtTA cells were cultured in Earle's Minimal Essential Medium (EMEM) with a final FCS concentration of 10 % v/v.

Lipopolyamines used in the cell viability assay were the liposomal formulations Lipofectin (DOTMA/DOPE 1:1 w/w) and Lipofectamine (DOSPA/DOPE 3:1 w/w) and the non-liposomal formulations Transfectam® (DOGS), N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine and N^4,N^9-dilinoleoyl spermine. They were used either complexed or as free lipopolyamine.

For DNA-vector complex formation, two solutions were prepared for each well (96-well plate), solution A (DNA) and solution B (condensing agent). Solution A was prepared by diluting DNA solution (0.2 µg/well) to 10 µl using Opti-MEM, a serum free media (Gibco), in a sterile 1.5 ml micro-centrifuge tube obtained from Eppendorf Ltd., gently mixed using a vortex, for 2 secs and incubated for 30 mins at room temperature. Solution B was prepared by diluting transfection agent solution to 10 µl/well using Opti-MEM, serum free media, in a sterile 1.5 ml micro-centrifuge tube, gently mixed using a vortex, for 2 secs and incubated for 30 mins at room temperature. The complex was then prepared by mixing solution A and B with

vortex for 1 sec then incubated for 20 mins at room temperature. Each transfection agent used in solution B was at a different volume according to the required N/P ratio for 0.2 μg DNA/well, as described before in the N/P determination section for EthBr assay.

For primary fibroblasts and carcinoma cells toxicity study, 8 x 10^3 cells/well were seeded in 96-well plates, obtained from NUNC, in 200 μl of 15 % v/v FCS EMEM and 10 % v/v FCS EMEM in the case of primary fibroblasts and HtTA, respectively. Cells were incubated at 37 °C and 5 % v/v CO_2 in a CO_2 incubator for 24 h to reach 50-60 % confluency. The adherent cells were washed twice with PBS solution, and then washed once with Opti-MEM. Washed medium was removed and replaced with 80 μl of Opti-MEM to which 20 μl of either the transfection complex (lipoplex), the free lipopolyamine, or the free DNA (naked DNA) was added to the plates except for control cells where 100 μl of Opti-MEM was added (no DNA complex solution). The plates were incubated for 4 h at 37 °C in an atmosphere of 5 % v/v CO_2. After that, the Opti-MEM in the incubated plates was aspirated and replaced with 200 μl of 15 % v/v FCS EMEM and 10 % v/v FCS EMEM in the case of primary fibroblasts and HtTA, respectively. Then, incubated for a further 44 h at 37 °C in an atmosphere of 5 % v/v CO_2.

After incubation for 44 h, the media was replaced with 90 μl of fresh media and 10 μl of sterile filtered MTT solution (Sigma-Aldrich) (5 mg/ml) to reach a final concentration of 0.5 mg/ml. Then the plates were incubated for a further 4 h at 37 °C in an atmosphere of 5 % v/v CO_2. After incubation, the media and the un-reacted dye were aspirated and the formed blue formazan crystals were dissolved in 200 μl/well of DMSO. The produced colour was measured using a plate-reader (VERSAmax™) at wavelength 570 nm. The % viability related to control wells containing cells without DNA and/or polymer and is calculated as follow (200):

$$\%\ cell\ viability = \left(\frac{A_{570}\ sample}{A_{570}\ control}\right) \times 100 \qquad [10]$$

Characterization of the formed nanoparticles

Particle size

The average particle size for the lipoplexes formed (at their optimum charge ratio of transfection), after mixing with a vortex mixer, was determined using a Malvern Zetasizer Nano S (Malvern Instruments) All measurements were carried out on lipoplexes with 5 μg/ml plasmid DNA in HEPES buffer at pH 7.4 and 20 °C.

For pEGFP lipoplex formation, two solutions were prepared for each well, solution A (DNA) and solution B (lipopolyamine). Solution A was prepared by diluting DNA solution (10 μg/ml) in sterile Falcon polypropylene tubes (15 ml), obtained from Falcon, gently mixed using a vortex for 2 secs, and then incubated for 30 mins at room temperature. Solution B was prepared by diluting the lipopolyamine solution to 1.0 ml using HEPES buffer, in sterile Falcon polypropylene tubes (15 ml), gently mixed using a vortex for 2 secs, and then incubated for 30 mins at room temperature. The complex was then prepared by mixing solutions A and B by vortex for 1 sec, and then incubated for 20 mins at room temperature.

Each lipopolyamine used in solution B was at a different concentration from the required N/P ratio for 5 μg DNA/ml as described before in the N/P determination section for EthBr assay. The charge ratio was calculated according to the ammonium/phosphate (+/-) charge ratio of the investigated lipopolyamines to DNA. For DNA it was calculated as 330 g/mole is DNA that contains one negative charge (5). In case as of N^4,N^9-distearoyl spermine, it was calculated as 735.7g/mole that contains two primary amino groups that are protonated (positively charged) at pH 7.4. For N^4,N^9-dioleoyl spermine it was calculated as 731.26 g/mole that contains two primary amino groups that are protonated (positively charged) at pH 7.4. N^4,N^9-Dilinoleoyl spermine was calculated as 727.7g/mole that contains two primary amino groups that are protonated (positively charged) at pH 7.4. In the case of the commercially available lipopolyamine (DOGS, Transfectam®) it was calculated as 1263 g/mole that contains three nitrogens that are protonated at pH 7.4. In the non-liposomal formulations, Transfectam® carries 3 positive charges (185).

The mean hydrodynamic diameter of the particles, d_h (from 10 measurements per sample) was computed from the intensity of the scattered light using the Malvern software package based on the theory of Brownian motion and the Stokes-Einstein equation (201).

$$D = \frac{kT}{3\pi\eta d_h} \qquad [11]$$

where D is the diffusion coefficient, k is the Boltzmann constant, T is the temperature, and η is the solvent viscosity.

Zeta potential

ζ-Potential measurements were determined using a Malvern Zetasizer Nano ZS (Malvern Instruments). All measurements were carried out on lipoplexes with 5 μg/ml plasmid DNA in HEPES buffer at pH 7.4 and 20 °C.

For pEGFP lipoplex formation, two solutions were prepared for each well, solution A (DNA) and solution B (lipopolyamine). Solution A was prepared by diluting DNA solution (10 μg/ml) in sterile Falcon polypropylene tubes (15 ml), gently mixed using a vortex for 2 secs, and then incubated for 30 mins at room temperature. Solution B was prepared by diluting the lipopolyamine solution to 1.0 ml using HEPES buffer, in sterile Falcon polypropylene tubes (15 ml), gently mixed using a vortex, for 2 secs and incubated for 30 mins at room temperature. The complex was then prepared by mixing solution A and B with vortex for 1 sec then incubated for 20 mins at room temperature.

Each lipopolyamine used in solution B was at a different concentration to the required N/P ratio for 5 μg DNA/ml, as described before in the N/P determination for the particle size and EthBr experimental sections. In the non-liposomal formulations, Transfectam® carries 3 positive charges (185), while N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine and N^4,N^9-dilinoleoyl spermine each carry 2 positive

charges. The ζ-potential measurements of the nanoparticles were computed from the electrophoretic mobility using the Malvern software package based on the Henry equation:

$$U_E = \frac{2\varepsilon\zeta f(\kappa a)}{3\eta} \qquad [12]$$

where U_E is the electrophoretic mobility (velocity of the nanoparticle in a unit electric field), ζ is the zeta potential, ε is the dielectric constant, η is the solvent viscosity and f (κa) is Henry's function, which in aqueous media and electrolyte is equal to 1.5 known as Smoluchowski approximation.

CHAPTER 3

Formulation and Delivery of DNA Condensed by a Synthetic Lipospermine, N^4,N^9-Dioleoyl Spermine

Introduction

Non-viral delivery systems can be defined to include the use of plasmid DNA alone (so-called "naked DNA") (10) as well as DNA complexed to synthetic carriers such as cationic lipids (202, 203) or polymers (204). The design of an efficient formula for the delivery of genetic material requires a detailed understanding of the mechanism of gene delivery to the nucleus. Since the design and formulation of Lipofectin by Felgner and co-workers, reported in 1987 (16), the focus on non-viral vectors for DNA delivery has shown a remarkable increase worldwide (205-207).

Efficient non-viral formulation should be able to deliver safely the required DNA across the various cellular barriers to the nucleus. Cationic lipids are considered to be the major gene carriers among the non-viral delivery systems. They have the ability to condense DNA into particles that can be readily endocytosed by cultured cells, and facilitate endosomal escape leading to efficient delivery to the nucleus (208). They can be classified as liposomal and non-liposomal non-viral delivery vectors. Liposomal delivery vectors usually contain two types of lipids, a cationic lipid (positively charged amphiphile) for DNA condensation and cellular membrane interaction, and a neutral helper lipid (phospholipid), most use dioleoylphosphatidyl-ethanolamine (DOPE) (Fig. 1) to increase transfection efficiency as it has a membrane fusion promoting ability (16, 108, 209). Non-liposomal cationic-lipid delivery vectors combine both the characteristics of cationic and helper lipids.

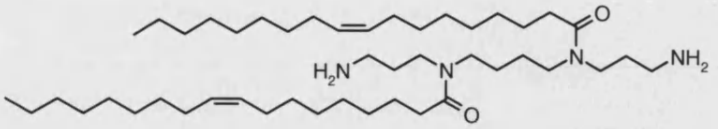
The synthesis of the lipopolyamine dioctadecylamidoglycylspermine (DOGS) by Behr and co-workers (179) as a promising transfecting agent, encouraged several laboratories to focus on the synthesis of novel cationic lipids based on the naturally occurring polyamine spermine e.g. RPR120535 (182) and 1,3-dioleoyloxy-2-(6-carboxyspermine) DOSPER (177) (Fig. 1). The design of a novel lipopolyamines formula for DNA condensation and cellular delivery relies on previous and continuing studies of the structure-activity relationships of DNA binding and condensation by polyamines (19, 171, 210, 211). Although lipopolyamines are less efficient in comparison with viral vectors, their promising lower toxicity than viral

vectors ensures a continuous effort to design novel lipopolyamines with improved transfection efficiency.

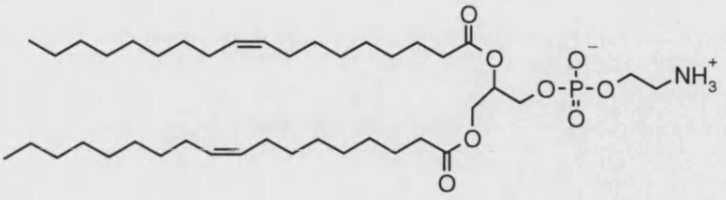
In this chapter, we have synthesized and formulated a novel lipospermine, in which a di-amine derived from spermine (the cationic moiety) and dioleoyl chains (the lipophilic moiety), that are reported to improve the transfection efficiency by fusion with cellular membrane (212), these unsaturated chains are linked by amide bonds at the secondary amino groups of spermine to form N^4,N^9-dioleoyl spermine. These amide linkers have the advantages of being both biodegradable and less toxic than the ether bonds in DOTMA (213, 214). N^4,N^9-Dioleoyl spermine was only previously reported by Kirby and his colleagues (187) who synthesized N^4,N^9-dioleoyl spermine as an intermediate for the synthesis of the gemini surfactants N^1,N^{12}-di(Lys-Lys-Lys-Ser)-N^4,N^9-dioleoylspermine) and N^1,N^{12}-di-Lys-N^4,N^9-dioleoylspermine). In the same work, similar spermine conjugates were synthesized by replacing the hydrocarbon chains position on spermine from the (N^4 and N^9) to (N^1 and N^{12}) with the basic amino acid residues position. Publications from their research work did not report any intention to use N^4,N^9-dioleoyl spermine as an NVGT vector (186, 187). Also, no other research group has reported the investigation of N^4,N^9-dioleoyl spermine as an NVGT vector.

The ability of this synthetic lipopolyamine to condense DNA was studied using ethidium bromide (EthBr) fluorescence quenching and light scattering assays. Transfection efficiency was studied in an immortalized cancer cell line (HtTA), and in primary skin cells (FEK4) for the first time. The results are compared with two commercially available (liposomal) transfection formulations, Lipofectin® and Lipofectamine™ that incorporate such oleoyl or oleyl (C18) chains. Lipofectamine™ is available as a mixture of the cationic lipid DOSPA and the neutral helper lipid DOPE as 3/1 w/w, respectively. While Lipofectin® is available as a mixture of the cationic lipid DOTMA and the helper lipid DOPE as 1/1 w/w, respectively (Fig. 1). The cytotoxicity of these compounds was studied in both primary skin and immortalised cancer cell lines using an MTT assay.

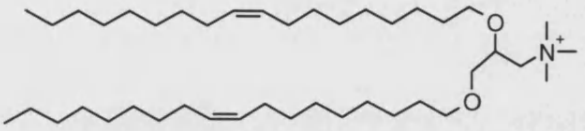
N^4,N^9-Dioleoyl spermine



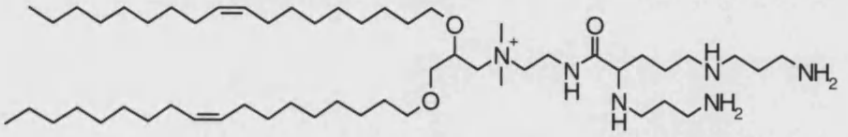
DOPE



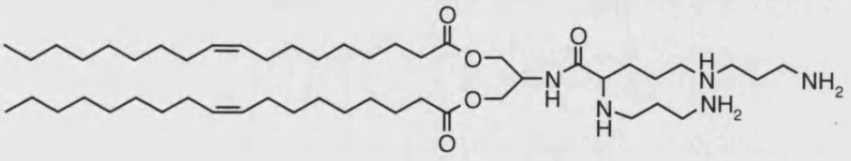
DOTMA



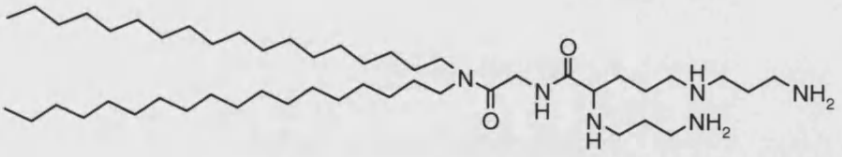
DOSPA



DOSPER



DOGS



RPR-120535

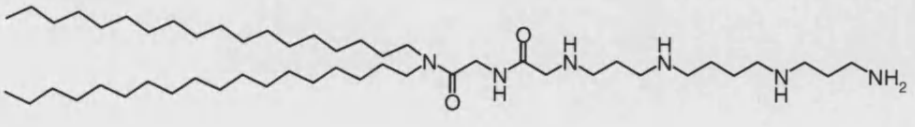


Figure 1. DOTMA, DOPE and spermine based lipopolyamines.

Synthesis of N^4,N^9-dioleoyl spermine

As indicated in the material and methods chapter, spermine was used as the starting material for the synthesis process (215), outlined in Fig. 2. Spermine was protected on the primary amino functional groups with ethyl trifluoroacetate (2.2 eq.) in methanol and the reaction mixture was stirred for 18 h at 25 °C. The solvent was evaporated to dryness in vacuo to form N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane, R_f 0.7 (CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v) with a yield of about 93 % as previously reported (216).

For oleic acid conjugation, dicyclohexylcarbodiimide (DCC, 2.5 eq), 1-hydroxybenzotriazole (HOBt, 0.2 eq) and oleic acid (2.2 eq) were added to the diprotected spermine solution (used without purification) in CH_2Cl_2 and methanol (1:1). The solution was stirred for 18 h at 25 °C. The solvent was evaporated to dryness in vacuo. The residue was dissolved in CH_2Cl_2 and the solution filtered and evaporated to dryness in vacuo to form N^4,N^9-dioleoyl-N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane.

For the removal of the di-trifluoroacetyl groups, the tetra-amide was dissolved in methanol and the pH of the solution was increased by saturating with ammonia gas, then it was left (18 h) and evaporated to dryness in vacuo to give a residue which was purified over silica gel (CH_2Cl_2-MeOH 5:3 v/v, then CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v) to afford N^4,N^9-dioleoyl spermine (Fig. 2) homogenous on silica gel thin-layer chromatography as a colourless oil (R_f 0.44) (CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v) with a 9 % overall yield, characterized by high resolution accurate mass spectroscopy (HRMS), $C_{46}H_{91}N_4O_2$ found (m/z $[M+H]^+$) 731.7115, requires 731.7142 (Δ ppm 3.7), $C_{45}{}^{13}CH_{91}N_4O_2$ found 732.7145, requires 732.7176 (Δ ppm 4.1).

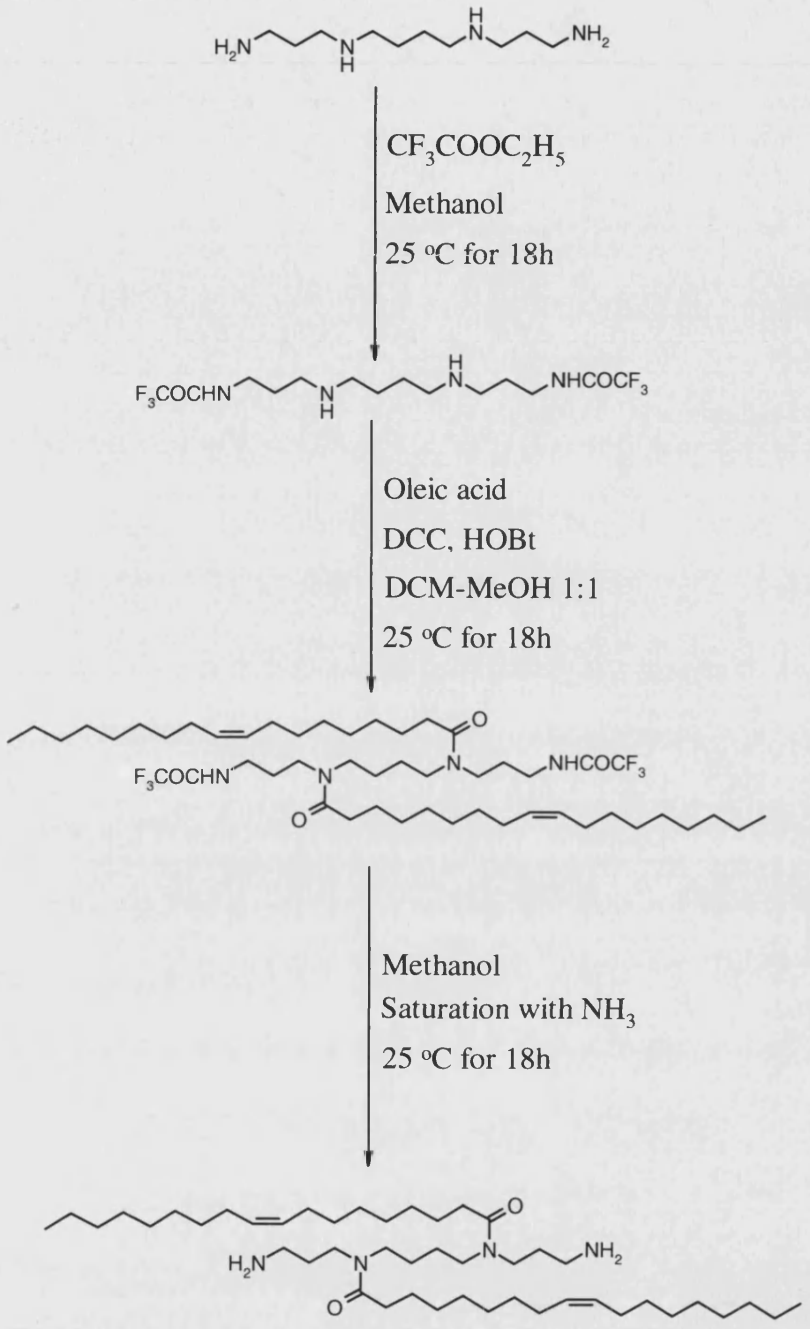


Figure 2. Synthesis of N^4,N^9-dioleoyl spermine.

DNA condensation studies

The use of an efficient carrier for nucleic acid delivery is considered to be a determinant factor for the successful application of gene therapy (217). This carrier is responsible for the complex process of gene delivery to the nucleus (26). Within the prerequisites for delivery of DNA across intact cytoplasmic membrane are condensation and masking the negative charge of the phosphate backbone. Condensation of DNA occurs when 90 % of the charge on DNA is neutralised (19, 22). To investigate the neutralization of the negative charges of the phosphate groups on the DNA back bone that leads to collapse of DNA into a more compact structure (nanoparticles). Two experiments were carried out, these are:

1. Ethidium bromide fluorescence quenching assay
2. Light scattering assay

The ability of the synthetic lipopolyamine N^4,N^9-dioleoyl spermine to condense both calf thymus DNA and circular plasmids DNA was investigated. Two plasmids were used in this study. pSV-β-galactosidase (pβ-gal, 6.8 kbp) with SV40 early promoter and enhancer that drive the transcription of *LacZ* gene which encodes the β-galactosidase enzyme. pEGFP encoding for enhanced green fluorescent protein (4.7 kbp) under CMV promoter. N^4,N^9-Dioleoyl spermine was compared with the natural polyamine spermine (Fig. 3). Also, the cationic polymers polyethylenimine (PEI) and poly-L-lysine (PLL) (Fig. 3) were used as model for their well known DNA condensation ability to compare with the lipopolyamine N^4,N^9-dioleoyl spermine ability to condense DNA. Also, the ability of our novel polyamine conjugate N^4,N^9-dioleoyl spermine to condense DNA, leading to the formation of nanoparticles (that are suitable for gene delivery), was compared with the commercially available liposomal transfection formulations Lipofectin® and Lipofectamine™.

Spermine **PLL**

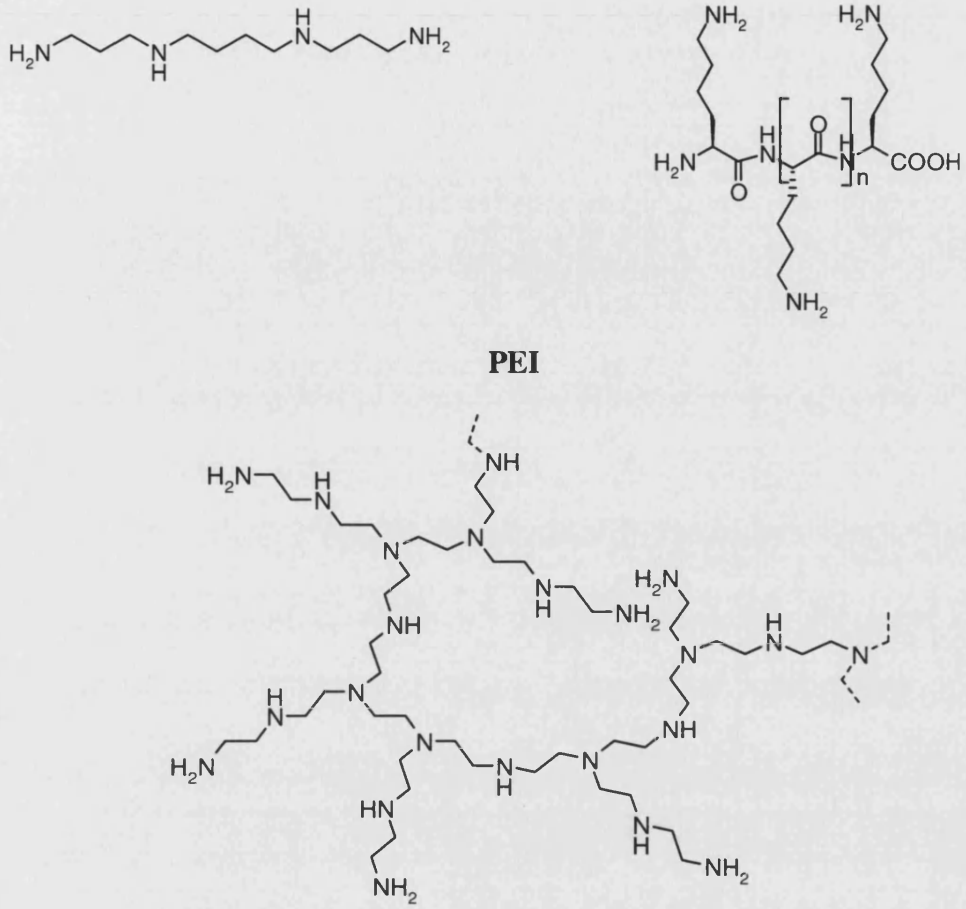


Figure 3. Spermine, PLL and PEI.

Ethidium bromide fluorescence quenching assay

Ethidium bromide (EthBr) (2,7-diamino-9-phenylphenanthridinium-10-ethyl bromide, Fig. 4) a cationic dye that displays a marked increase in the fluorescence upon binding with DNA and RNA through the intercalation between the EthBr phenanthridinium moiety and adjacent base pairs of specific DNA sequences (193, 218-220). The increase in the fluorescence of EthBr upon binding with DNA making EthBr a useful probe to measure drug-DNA interactions (193, 218). The binding of cationic molecules with DNA is not entirely responsible for the release of EthBr, but alteration of the molecular flexibility of DNA through cationic compaction facilitated the release of bound EthBr (193, 221, 222).

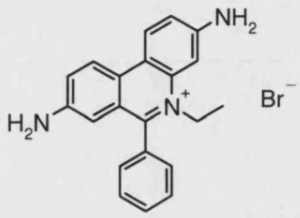


Figure 4. Structure of ethidium bromide.

The significant enhancement of fluorescence when EthBr intercalates DNA is attributed to the hydrophobic environment surrounding the EthBr molecule leading to longer life time for the excited state (218, 223). On the other hand, free EthBr (in solution) is strongly quenched by aqueous solvent, so it exhibits a weak fluorescence relative to that from intercalated dye (223). The fluorescence intensity of free EthBr (no DNA) in HEPES buffer solution (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was usually small (background). On the other hand, the fluorescence intensity of the EthBr solution was significantly increased after the addition of DNA solution, as a result of EthBr intercalation between DNA base-pairs. The fluorescence was expressed as the percentage of the maximum fluorescence when EthBr was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free EthBr in solution.

We started DNA condensation studies through investigating the effect of spermine to condense calf thymus DNA, plasmid β-galactosidase, pEGFP and the synthetic polynucleotides poly(dA-dT).poly(dA-dT) and poly(dG-dC).poly(dG-dC). The charge ratio was calculated according to the ammonium/phosphate (+/-) charge ratio for spermine as 202.35 g/mole is spermine that contains four nitrogens that can be protonated. Spermine has a pK_a values of 10.9, 10.1, 8.9 and 8.1, and according to Henderson-Hasselbach equation, spermine has 3.8 charges / molecule at pH 7.4 (145).

From these results, it was found that spermine caused a decrease in the fluorescence intensity with the studied polynucleotides. In the case of the synthetic polynucleotides poly(dA-dT).poly(dA-dT) and poly(dG-dC).poly(dG-dC) there is a

lower decrease in fluorescence intensity of EthBr-poly(dG-dC).poly(dG-dC) complex in comparison with the fluorescence intensity of EthBr-poly(dA-dT).poly(dA-dT) complex (Fig. 5) which may be attributed to the bending of poly(dA-dT) major groove interacted with spermine that leads to widening of minor groove of the alternating A-T sequences that increase EthBr mobility in this minor groove which is not the case of poly(dG-dC) due to stiffness of the structure due to the presence of more hydrogen bonds (222, 224). These results were in accordance with the previous results reported by Delcros, Marton and co-workers (222).

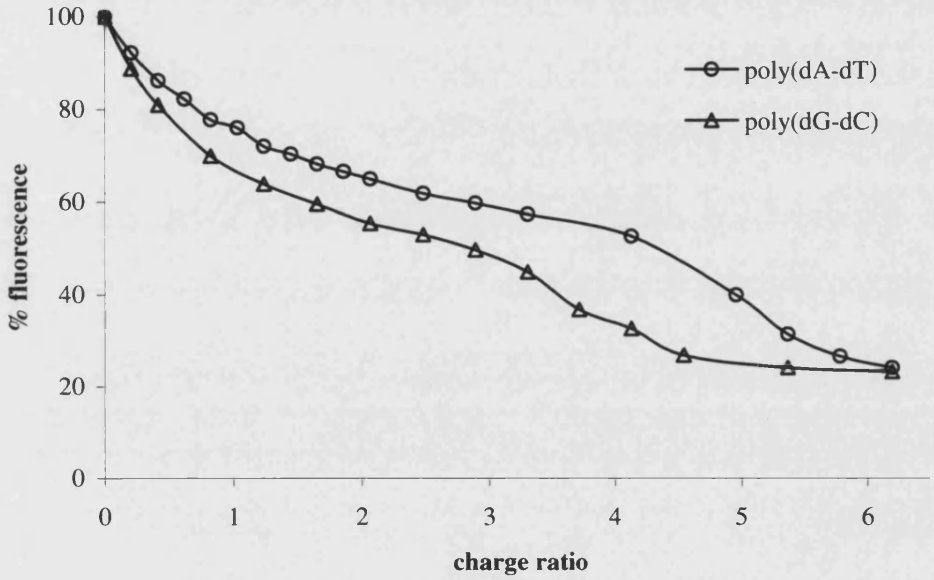


Figure 5. Plot of EthBr displacement assay of spermine with poly(dA-dT).poly(dA-dT) and poly(dG-dC).poly(dG-dC).

Fig. 6 shows the decrease in fluorescence intensity of EthBr for both calf thymus DNA and pβ-gal interacted with spermine. These results show the ability of spermine to condense, both linear calf thymus DNA and circular plasmid DNA through quenching of EthBr fluorescence by up to 17 % and 23 %, respectively, at charge ratio 6. Also, in the case of the interaction of spermine with calf thymus DNA and plasmid β-galactosidase, there is no significant difference in the decrease of the fluorescence intensity.

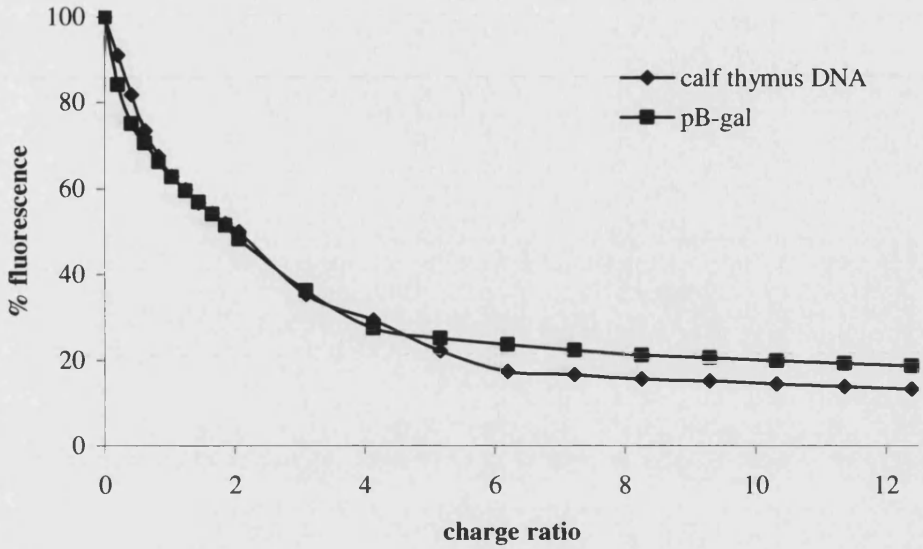


Figure 6. Plot of EthBr displacement assay of calf thymus DNA and pβ-gal with spermine at different N/P charge ratios.

To study the ability of poly-L-lysine (PLL) (225) and polyethylenimine (PEI) (95) (Fig. 7) to condense calf thymus DNA as well as compared this effect with the effect of spermine to condense DNA. In the case of polyethylenimine, two different molecular weights were investigated, PEI average molecular weight 2,000 Da (PEI 2k) and 60,000 Da (PEI 60k). In the case of PLL, two different molecular weights were investigated, PLL average molecular weight 9,600 Da (PLL 9.6k) and 27,000 Da (PLL 27k). In the case of PEI 2k and PEI 60k, the charge ratio was calculated by assuming that 43.1 g/mol is the repeating unit of PEI that contains one nitrogen atom that can be protonated (75, 113).

It seems to be that there is no agreement in the literature for the calculation of the PEI/DNA charge ratio. While it is calculated as PEI nitrogen/DNA phosphate (95) not as charge ratio, it is reported that every third nitrogen atom of PEI polymer is a protonatable nitrogen (95), but one in five of the protonatable nitrogens in PEI are protonated at pH 7 (226). PEI charge ratio was calculated as 25 % of the amino groups in the polymer that can be protonated at pH 7.4 (75, 113). However, Wagner (206) reported that one of every seven nitrogens within PEI polymer is protonated at pH 7. In our studies, the charge ratio of PEI 2k and PEI 60k were calculated by multiplying the weight ratio of PEI to DNA by 1.9 (75). The value 1.9 indicated that

25 % of the amino groups in the polymer are protonated at pH 7.4. In the case of PLL 9.6k and PLL 27k, the charge ratio was calculated as one nitrogen (as one positive charge) that can be protonated/lysine monomer.

The results in Fig. 7 show that the studied polyamines, PEI and PLL polycations, are able to condense and displace EthBr from DNA efficiently at a significantly low charge ratio compared to spermine. These results show that the efficiency of DNA condensation was in the order of PEI > PLL > spermine according to the charge ratio. PEI are the most efficient by completely condensing DNA (less than 90 % fluorescence quenching) at a lower charge ratio, PEI 2k completely condense calf thymus DNA (less than 10 % residual fluorescence intensity) at charge ratio 0.3, and PEI 60k completely condense calf thymus DNA (less than 90 % fluorescence quenching) at charge ratio (N/P) 0.2 (Fig. 7). PLL 9.6k and PLL 27k, displace intercalated EthBr from calf thymus DNA by less than 90 % fluorescence quenching at charge ratios 1.8 and 1.3, respectively. The tetra-amine spermine shows lower ability in DNA condensation in comparison with the polycations PEI and PLL, through quenching of EthBr fluorescence up to 17 % at charge ratio (N/P) 6.

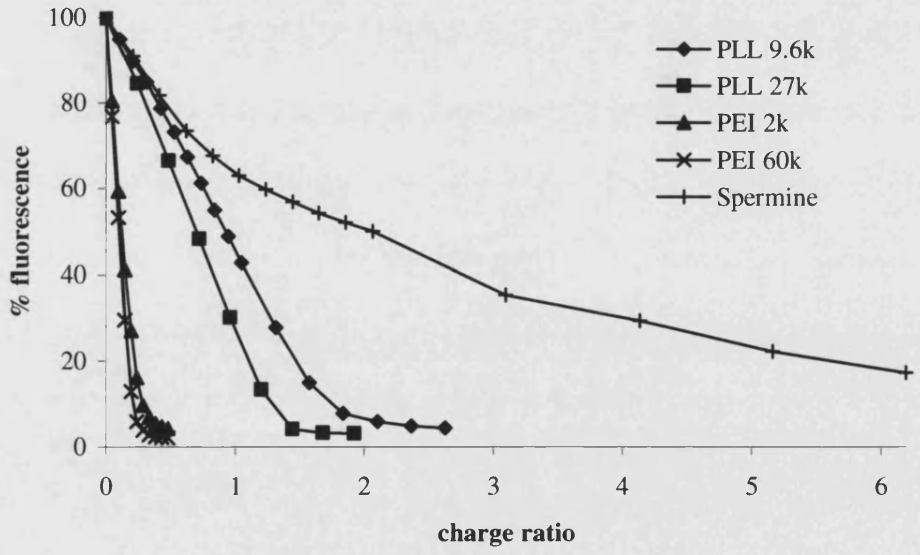


Figure 7. Plot of EthBr fluorescence quenching assay of calf thymus DNA with PLL 9.6k, PLL 27k, PEI 2k, PEI 60k and spermine.

These results confirmed that, both the number of positive charges and their distribution on the surface of the molecule have profound effects on DNA condensation as indicated in refs (49, 210, 227). Also, spermine is not considered as an efficient condensing agent. Although PEI and PLL show more efficiency in DNA condensation, but their well known toxicity limits their use in DNA formulation as non-viral gene delivery vectors (228, 229).

On studying the effect of each condensing agent on different DNAs, calf thymus DNA and herring testes DNA were used as an example of linear DNA and plasmid β-gal was used as an example of circular plasmid DNA. These results showed that there is no significant variation in the condensation ability of the studied polyamines on calf thymus DNA and herring testes DNA using PEI 60k (Fig. 8A), PEI 2k (Fig. 8B) or PLL 27k (Fig. 8C). Also, there is no significant difference between calf thymus (linear) DNA and plasmid β-galactosidase using PLL 9.6 as indicated in Fig. 8D. Similarly, in the case of the interaction of spermine with calf thymus DNA and plasmid β-galactosidase, there is no significant difference in the decrease of the fluorescence intensity (Fig. 6).

The ability of the synthetic N^4,N^9-dioleoyl spermine to condense calf thymus DNA and pEGFP as well as compared this effect with the effect of different known polycations spermine, PLL (225) and PEI (95) (Fig. 9) for DNA condensation was investigated using EthBr fluorescence quenching assay. Charge ratio was calculated as two positive charges/ N^4,N^9-dioleoyl spermine molecule (731.2 g/mol) at pH 7.4. The binding ability was in the order PEI > N^4,N^9-dioleoyl spermine > PLL > spermine according to the charge ratio.

On the other hand, the results revealed that N^4,N^9-dioleoyl spermine is able to condense DNA at a lower charge ratio than PLL 9.6K and spermine (Fig. 9), and produces a 50 % fluorescence decrease at charge ratio 0.52. These results showed the ability of the cationic lipid N^4,N^9-dioleoyl spermine (derived from spermine by conjugation with two oleoyl moieties on the secondary amino groups of spermine) to condense DNA efficiently compared to the natural polyamine spermine and the efficient DNA condensing agents, PLL and PEI cationic polymers, that are used as model for DNA condensation in this study.

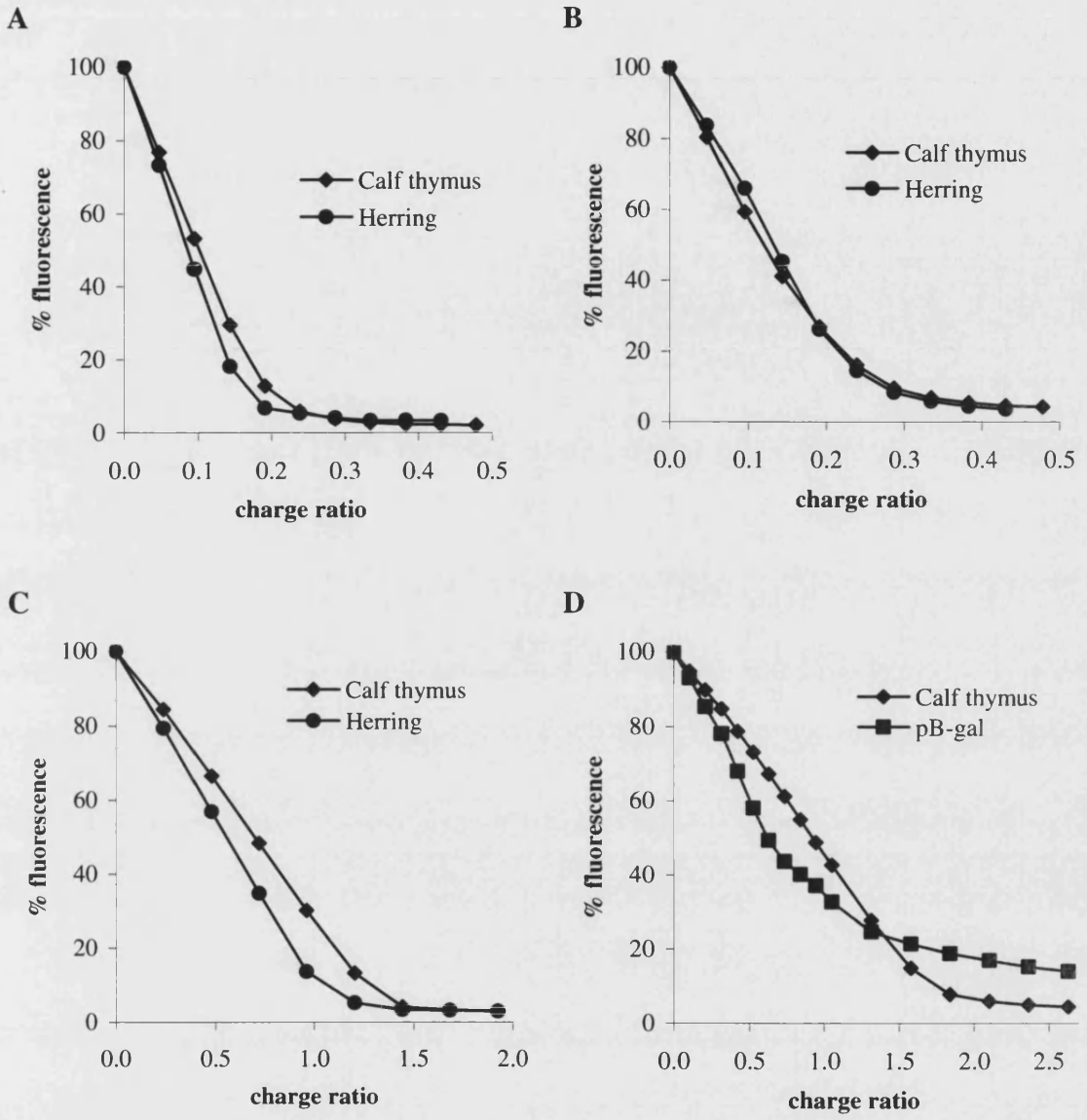


Figure 8. Plot of EthBr displacement assay of **A** PEI 60k, **B** PEI 2k, **C** PLL 27k with calf thymus DNA and herring DNA, and **D** PLL 9.6k with p-β-galactosidase and calf thymus DNA.

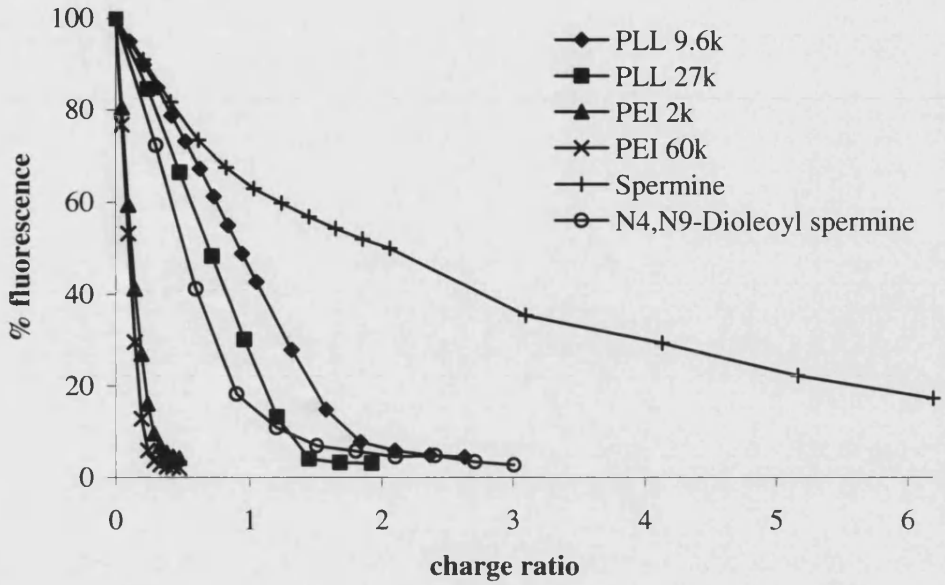


Figure 9. Plot of EthBr fluorescence quenching assay of calf thymus DNA with PLL 9.6K, PLL 27K, PEI 2K, PEI 60K, spermine and N^4,N^9-dioleoyl spermine.

To quantify the ability of the investigated polyamines in condensing DNA, the binding constant of these polyamines with DNA was estimated (Table 1) and compared using EthBr assay that has been shown to be a valid method for comparison of DNA binding affinity (144, 194, 221). The drug concentration producing 50 % inhibition of fluorescence is approximately inversely proportional to the binding constant (194).

From Table 1 data, the concentrations of condensing agents producing 50 % fluorescence inhibition were calculated (equation 1) as shown in the experimental chapter. The "apparent" equilibrium constant (K_{app}) of polyamine binding was calculated taking into account that the binding constant of ethidium to be 10^7 M^{-1} (194) and the concentration of EthBr is 1.3 μM. From these results it was found that PEI 60k has the highest binding affinity (K_{app} $6.5x10^9$ M^{-1}) between the compared polyamines and spermine the lowest with binding affinity (K_{app} $4x10^6$ M^{-1}) which could be attributed to the higher number of positive charges and the charge distribution on PEI 60k compared with spermine.

$$K_b \text{ (vector)} = \frac{[EthBr]\times 10^7}{[vector]} \qquad [1]$$

Table 1. Calf thymus DNA binding data

Condensing agent	Drug concentration at 50 % fluorescence quenching (μM)	K_{app}*
Spermine	3.295	$4x10^6$ M^{-1}
PLL 9.6k	0.125	10^8 M^{-1}
PLL 27k	0.028	$4.6x10^8$ M^{-1}
PEI 2k	0.063	$2.1x10^8$ M^{-1}
PEI 60k	0.002	$6.5x10^9$ M^{-1}
N^4,N^9-dioleoyl spermine	1.7	$0.8x10^7$ M^{-1}

* "apparent" equilibrium binding constant of condensing agent to calf thymus DNA

Also, to extend our investigation to validate the ability of our novel polyamine conjugate N^4,N^9-dioleoyl spermine to condense DNA, condensation results were compared with two commercially available cationic lipid (liposomal) transfection formulations, Lipofectin® (DOTMA/DOPE 1/1 w/w) and Lipofectamine™ (DOSPA/DOPE 3/1 w/w). Fig. 10 shows DNA condensation ability of N^4,N^9-dioleoyl spermine in comparison with the commercially available, cationic lipid formulations Lipofectin® and Lipofectamine™. All three cationic lipid formulations have the ability to condense completely DNA through the displacement of EthBr leading to fluorescence quenching. At lower charge ratios, N^4,N^9-dioleoyl spermine has better ability to suppress fluorescence, which produces 50 % fluorescence decrease at N/P charge ratio 0.52, than Lipofectin®, which produces 50 % fluorescence quenching at charge ratio 2.0 and Lipofectamine™ which produces 50 % fluorescence decrease at charge ratio 1.0. Also, Lipofectin was able to completely condense DNA (less than 10 % fluorescence quenching) at charge ratio 4.5, while Lipofectamine at charge ratio 1.8 and N^4,N^9-dioleoyl spermine at charge ratio 1.4.

On studying the effect of N^4,N^9-dioleoyl spermine on the type of DNA (calf thymus DNA, and plasmid pEGFP), it was found that there is no significant variation

in the condensation ability of the studied lipopolyamine on the type of DNA as shown in Fig. 11. These results are also in agreement with the previous results that were carried out by interaction of linear and circular plasmid DNA with the cationic polymers, PEI and PLL, and spermine (Figs. 6 and 8).

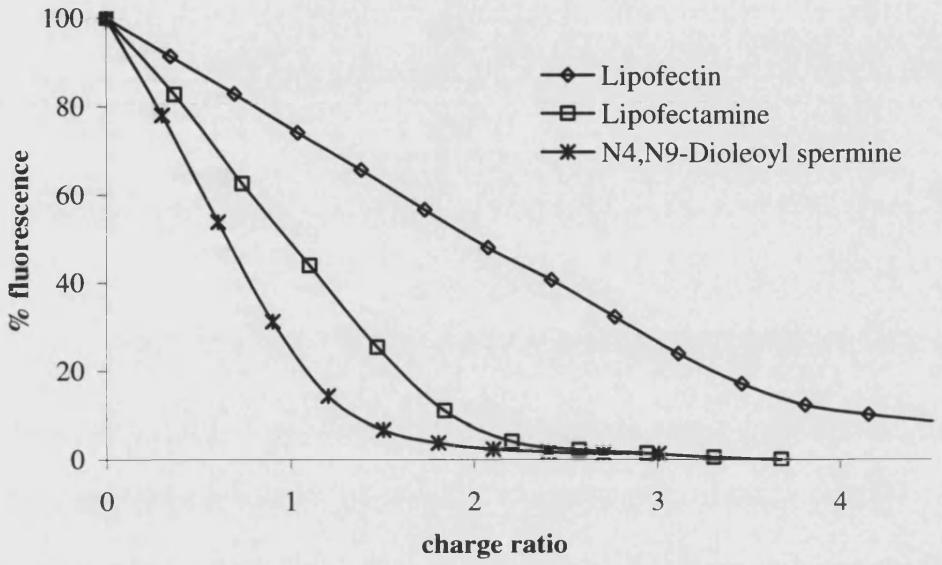


Figure 10. Plot of EthBr displacement assay of calf thymus DNA complexed with N^4,N^9-dioleoyl spermine, Lipofectin® and Lipofectamine™.

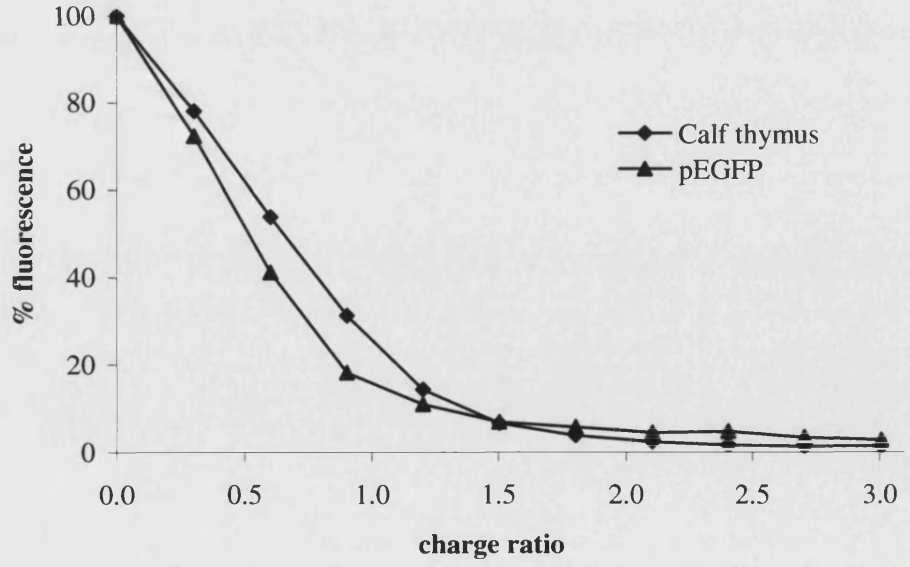


Figure 11. Plot of EthBr fluorescence quenching assay of N^4,N^9-dioleoyl spermine with calf thymus DNA and pEGFP.

EthBr fluorescence quenching results show the ability of the cationic lipid N^4,N^9-dioleoyl spermine to compact DNA more efficiently than both spermine and the powerful condensing agent cationic polymer PLL (Fig. 10). These results show the importance of the lipid moieties that have been bound to the cationic polyamine in order to achieve improvements in its ability to condense DNA, cellular entry, and lowering the toxicity of the polyamine conjugate (69, 206, 230).

DNA is condensed by neutralizing the DNA phosphate negative charges by the two primary amines that are positively charged at pH 7.4 and coated by the dioleoyl lipophilic moiety of N^4,N^9-dioleoyl spermine. In addition, the formation of N^4,N^9-dioleoyl spermine-DNA lipoplex at lower charge ratio decreases the toxicity of the DNA delivering lipopolyamine. In addition, as the mammalian cell membrane is a semi-permeable membrane formed of phospholipids bilayer that allows the transport of macromolecules by endocytosis, neutralization of the negative charges on the DNA by polycations will improve the delivery of DNA through the cell membrane because of the presence of negative charges on both DNA and cell membrane. Also, the positively charged lipid complex will mediate transfection by fusion with cell membrane (69, 93). These results also showed that both the number of positive charges and their distribution on the surface of the molecule have profound effects on DNA condensation, which are also in agreement with the published literature (49, 210, 227).

In addition, in a study on the transfection activity of cholesterol carbamate cationic lipids, Blagbrough and co-workers reported (210) that the carbamate with a spermine polyamine moiety has the highest transfection activity with its ability to condense DNA efficiently. Our findings are in agreement with the literature (210), the cationic liposomal formulation Lipofectin®, with its cationic moiety DOTMA containing one positively charged quaternary ammonium group, has lower ability to displace the EthBr from DNA than Lipofectamine™ formulation that contains DOSPA (Fig. 1) with its four positively charged nitrogens (185) and N^4,N^9-dioleoyl spermine with its two positively charged nitrogens.

The higher ability of N^4,N^9-dioleoyl spermine (two positive charges) over Lipofectamine™ (four positive charges) in DNA condensation, though N^4,N^9-dioleoyl

spermine has a lower number of positive charges/molecule, may be attributed to the distribution of the positive charges on the molecule allowing a higher affinity of the vector for DNA and leading to the efficient displacement of EthBr. Another variable is the liposomal formulation of Lipofectamine™ compared to the non liposomal formulation of N^4,N^9-dioleoyl spermine, where the addition of the neutral helper lipid DOPE to the liposomal formulae could affect the condensation ability of the cationic lipid used in the formulation.

Light scattering assay

This experiment has been carried out to investigate the condensation of DNA by polyamines and the formation of particles (20). The apparent UV absorbance at 320 nm (where there is no DNA absorbance above 300 nm) was measured (21, 210, 231) showing light scattering. The formation of DNA nanoparticles causes scattering of light that reduces light intensity at the UV detector. The results from Fig. 12 indicated the formation of particles upon interaction of spermine, PLL 9.6K, PLL 27k PEI 2K and PEI 60K.

Fig. 13 shows the increased in apparent absorbance by increased light scattering of the formed particles as a result of interaction of N^4,N^9-dioleoyl spermine with calf thymus DNA. The maximum apparent absorption was reached at a charge ratio of 1.7.

Also, light scattering and particle formation results of the lipoplex formed by interaction of N^4,N^9-dioleoyl spermine with calf thymus DNA were compared with the results of the interaction of calf thymus DNA with PLL, PEI and spermine (Fig. 14). These results indicate improved ability of N^4,N^9-dioleoyl spermine to condense DNA and form particles over PLL 9.6K, PLL 27K and spermine, which were also similar with the results concluded from EthBr fluorescence quenching study (Fig. 9).

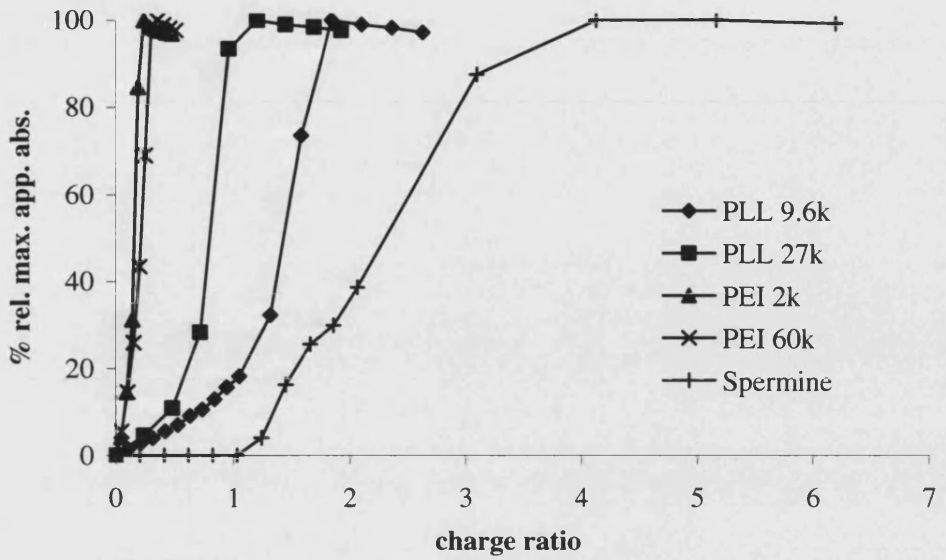


Figure 12. Light scattering assay (relative maximum apparent absorbance at λ = 320 nm) of calf thymus DNA with PLL 9.6k, PLL 27k, PEI 2k, PEI 60k and spermine.

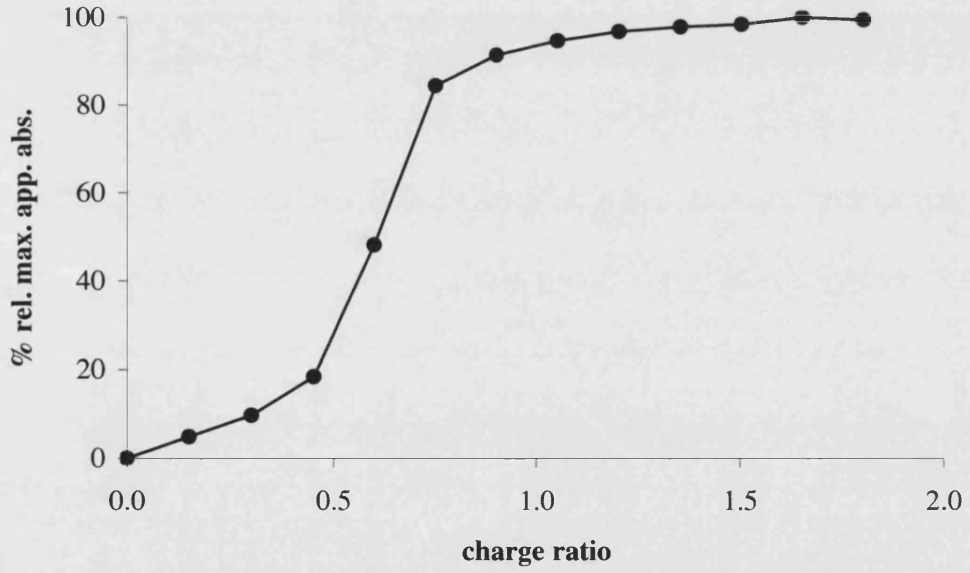


Figure 13. Light scattering assay (relative maximum apparent absorbance at λ = 320 nm) of calf thymus DNA with N^4,N^9-dioleoyl spermine.

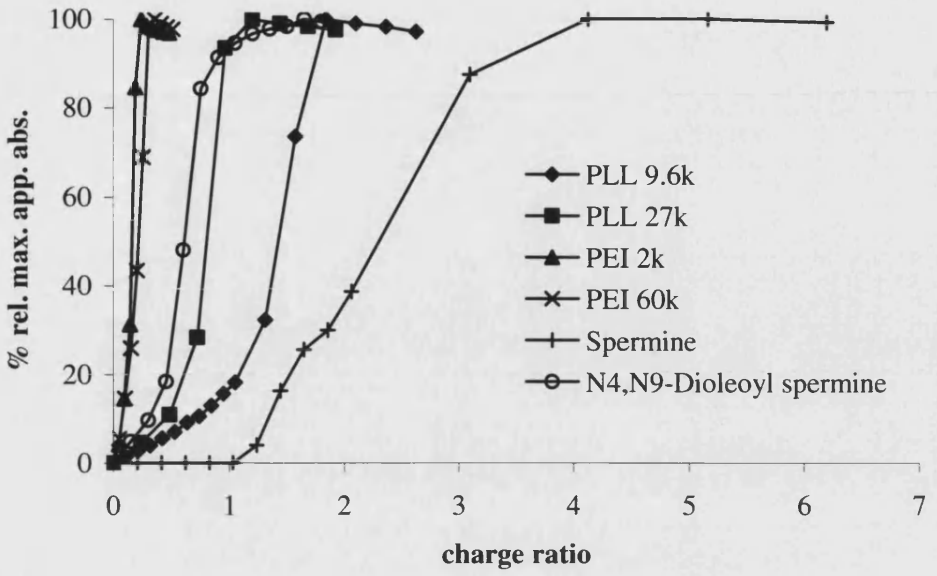


Figure 14. Light scattering assay (relative maximum apparent absorbance at $\lambda = 320$ nm) of calf thymus DNA with PLL 9.6k, PLL 27k, PEI 2k, PEI 60k, spermine and N^4,N^9-dioleoyl spermine.

In addition, Fig. 15 shows that the light scattering due to particle formation increases with the increase in the displaced EthBr and reaches the maximum at approximately the same charge ratio at which there is a maximum EthBr displacement, although the concentration of DNA used in light scattering experiments is ten times the concentration used in fluorescence quenching experiments which is related to the lack of sensitivity of the light scattering experiment in comparison with EthBr fluorescence assay.

Also, from light scattering results, there is a decrease in the % relative maximal apparent absorbance (% rel. max. app. abs.) after reaching the maximum absorbance, which could be attributed to the formation of polyamine-DNA aggregates as reported by Gosule and Schellman (232) where any DNA intra-chain binding agents will also act as inter-chain segment binders. The formation of aggregates at high charge ratio, at the relatively high concentration of DNA in comparison with EthBr assay, will decrease the scattering light that will decrease the apparent absorption value.

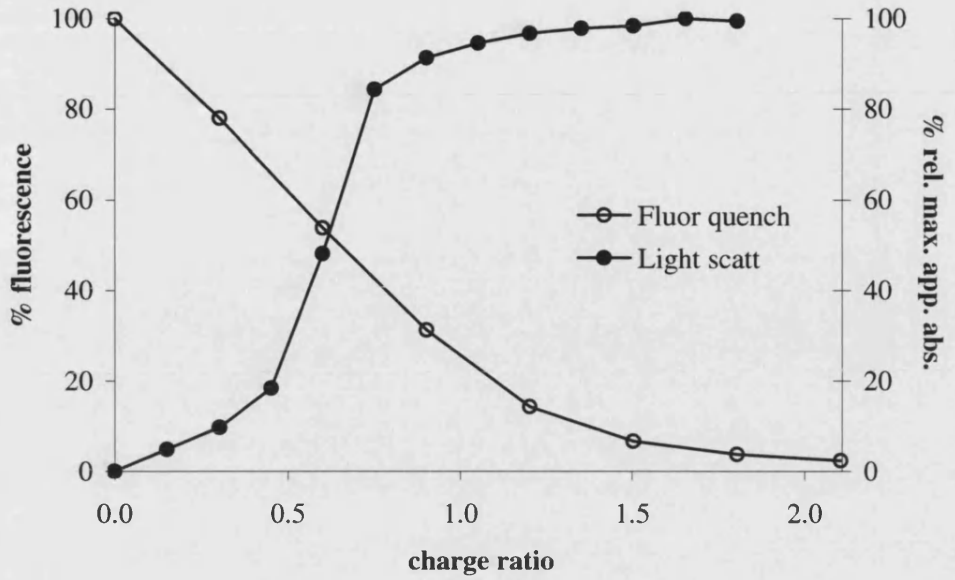


Figure 15. Comparison of EthBr displacement and light scattering assays of calf thymus DNA with N^4,N^9-dioleoyl spermine.

Transfection experiments

Following to the promising DNA condensation results of N^4,N^9-dioleoyl spermine shown in Figs. 9 and10, the ability of the lipopolyamine to deliver DNA efficiently to living cells was investigated. Two cell lines have been used in the transfection experiments in this study, primary skin fibroblast cells, FEK4 derived from an infant foreskin explant (passage dependent cell line) and human cervix carcinoma, HeLa derivative and transformed cell line (HtTA) was used as a model for carcinoma cells (an immortal cell line).

Transfection results and cell viability studies are considered indicative measures of the ability of the lipopolyamine to be used for in-vivo investigation of non-viral gene delivery vectors. N^4,N^9-Dioleoyl spermine transfection results were compared with two commonly used and commercially available transfection formulations Lipofectin® and Lipofectamine™. Both are available as liposomal formulations Lipofectin® (DOTMA/DOPE 1/1 w/w) and Lipofectamine™ (DOSPA/DOPE 3/1 w/w).

Transfection experiments were carried out using pEGFP, encoding for enhanced green fluorescent protein as a reporter gene, either complexed with DNA condensing agents, or free (naked DNA) as a negative control. DNA was used at a concentration of 2 μg/well. The transfection efficiency of the delivered pEGFP, i.e. levels of green fluorescent protein (EGFP positive cells) in the transfected cells, were detected using a fluorescence activated cell sorting (FACS) machine (λ_{ex} = 480 nm and λ_{em} = 530 nm). Transfection efficiency was expressed as % transfection representing % of EGFP-containing fluorescent cells. N^4,N^9-Dioleoyl spermine and both the commercially available liposomal formulations were used according to the required N/P ratio for 2 μg DNA/well, as described before in the N/P determination and the same protocol for transfection as mentioned in the materials and methods chapter.

Primary skin cell transfection studies

Primary cell lines are characterized as difficult to transfect cell lines (233, 234). Promising primary cell transfection results were achieved by electroporation technique where DNA is directly delivered to the nucleus (233, 234). Electroporation is used to overcome both extracellular and intracellular barriers that hinder a successful delivery of the gene to the nucleus. FEK4 cells were cultured in Earle's Minimal Essential Medium (EMEM) with foetal calf serum (FCS) serum concentration of 15 % v/v. The primary cells were seeded at a concentration of 1 x 10^6 cells/flask (T_{150}) that have 25 ml culture media and were passaged by trypsinisation technique.

Fig. 16 shows the transfection results of pEGFP into FEK4 cells using Lipofectin at different charge ratios. Results show that Lipofectin achieves a maximum of only 20 %. Lipofectin is unable to deliver DNA to primary cells efficiently. Also, at charge ratios higher than 1.2 there was a marked decrease in the number of cells due to toxic effects of the cationic lipid that was not sufficient for running the FACS experiment.

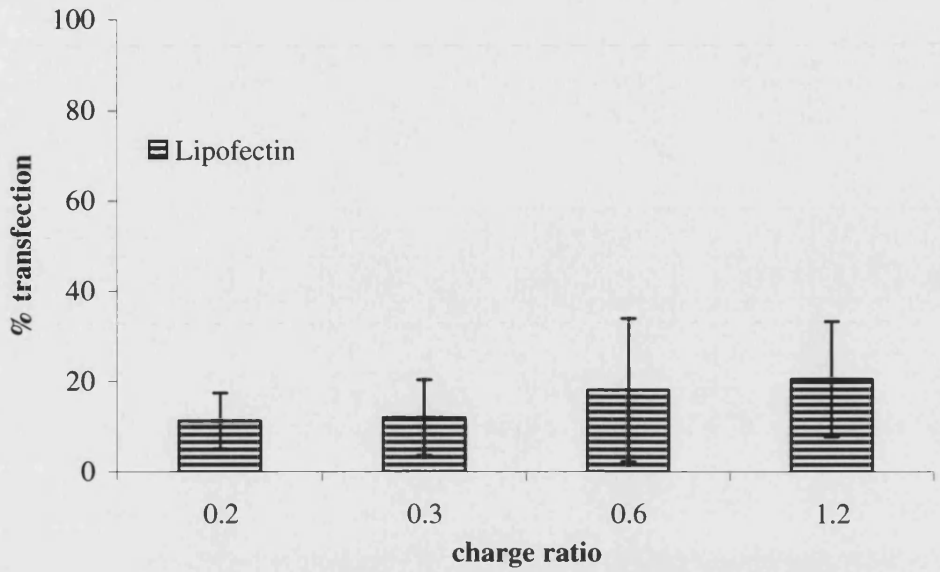


Figure 16. Lipofection of FEK4 cells transfected with pEGFP complexed with Lipofectin at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

Lipofectamine was investigated at different charge ratios in order to identify the optimum charge ratio for transfection. The results in Fig. 17 show that increasing the charge ratio will improve the transfection efficiency up to charge ratio 3.7 where there is no significant increase in the transfection efficiency. In addition Lipofectamine achieves higher transfection efficiency than Lipofectin formulation. Lipofectamine achieves a transfection efficiency of about 67% and 70% at charge ratios 3.7 and 7.3 respectively.

The transfection results of pEGFP into FEK4 using N^4,N^9-dioleoyl spermine indicated a high transfection ability of N^4,N^9-dioleoyl spermine (around 75 %) at charge ratios 2.5, 5.0,and 10. Also, there is no significant increase in the transfection ability of the lipoplex above charge ratio 2.5, as indicated in Fig. 18. The non-liposomal formulation N^4,N^9-dioleoyl spermine achieves higher transfection efficiency (75%) than the liposomal formulation Lipofectin (20%).

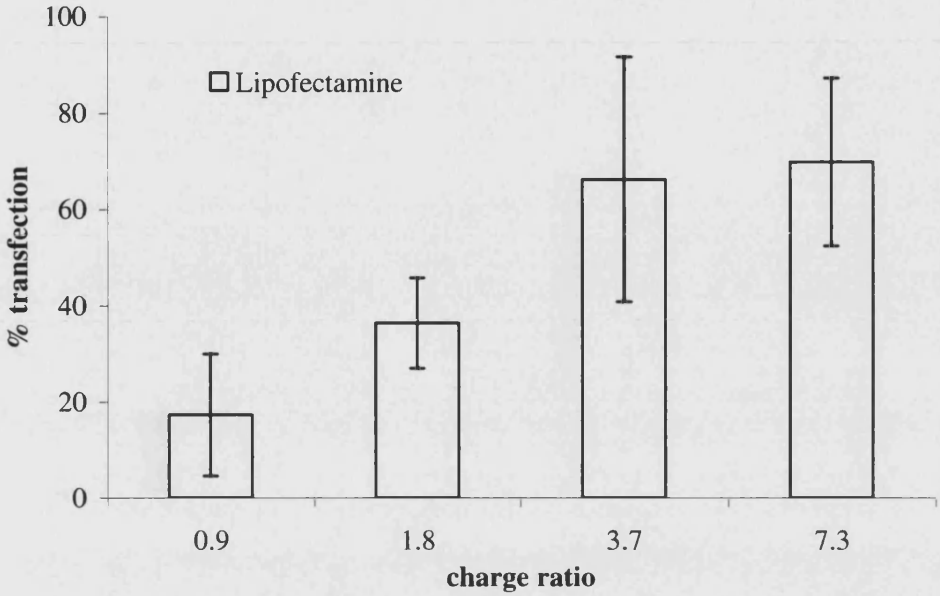


Figure 17. Lipofection of FEK4 cells transfected with pEGFP complexed with Lipofectamine at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

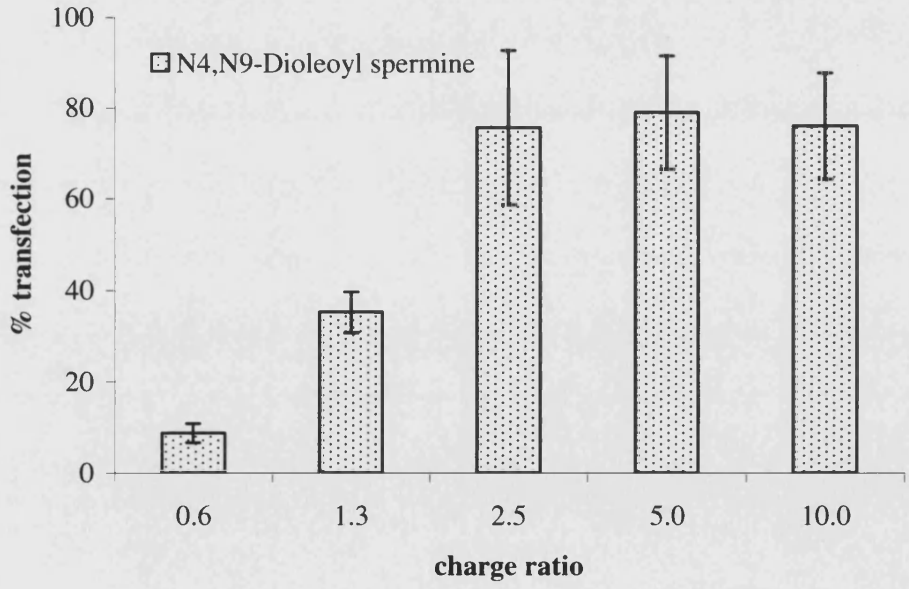


Figure 18. Lipofection of FEK4 cells transfected with pEGFP complexed with N^4,N^9-dioleoyl spermine at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

Cancer cell line transfection studies

HtTA cells used in this study are a HeLa derivative and transformed cell line. The cancer cell line Hela cells are the first continuous cancer cells (cervical cancer cells) isolated in 1951 from the cervix of a 30-year-old mother lady called Henrietta Lacks, at The Johns Hopkins Hospital in Baltimore, Maryland by George Gey (235). The HtTA cells being stably transfected with a tetracycline-controlled transactivator (tTA) consisting of the tet repressor fused with the activating domain of virion protein 16 of the herpes simplex virus (HSV) (236). The tTA sequence stimulates transcription from a minimal promoter sequence and it is sensitive to the concentration of tetracycline (236, 237). HtTA cells were cultured in EMEM supplemented with FCS 10 %, penicillin and streptomycin (50 IU/ml each), glutamine (2 mM), and sodium bicarbonate (0.2 % w/v).

Transfection results of HtTA with the lipoplex (Lipofectin®-pEGFP) at different charge ratios show increase in the transfection efficiency by increasing the charge ratio (Fig. 19). Lipofectin shows transfection efficiency more than 50 % at charge ratios 0.3, 0.6 and 1.2. Lipofectin achieves higher levels of EGFP expression in the case of cancer cell line HtTA compared to the primary cell line FEK4 transfection results.

Also, in the case of the commercially available liposomal formulation Lipofectamine™ with pEGFP to form lipoplexes at different charge ratios show increase in the transfection efficiency by increasing the charge ratio (Fig. 20). The increase in the transfection efficiency is up to charge ratio 3.7 where there is no significant improvement in the transfection efficiency at higher charge ratios.

The same results were achieved with the lipofection of the non-liposomal N^4,N^9-dioleoyl spermine-pEGFP lipoplexes at different charge ratios. These results show increase in the transfection efficiency by increasing the charge ratio up to charge ratio 2.5 (75 %) (Fig. 21). There is no significant increase in the transfection efficiency at higher charge ratios (5.0 or 10.0).

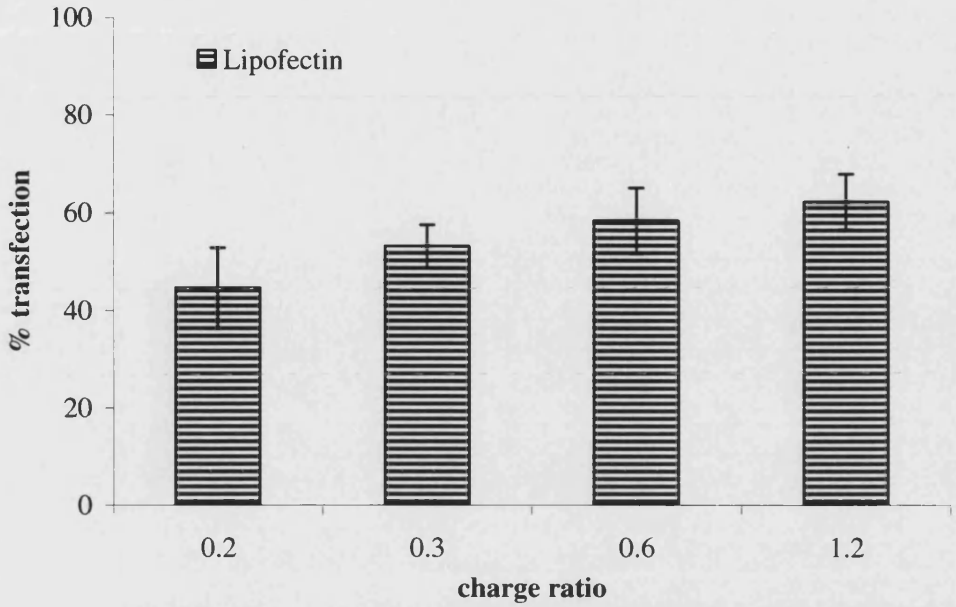


Figure 19. Lipofection of FEK4 cells transfected with pEGFP complexed with Lipofectin at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

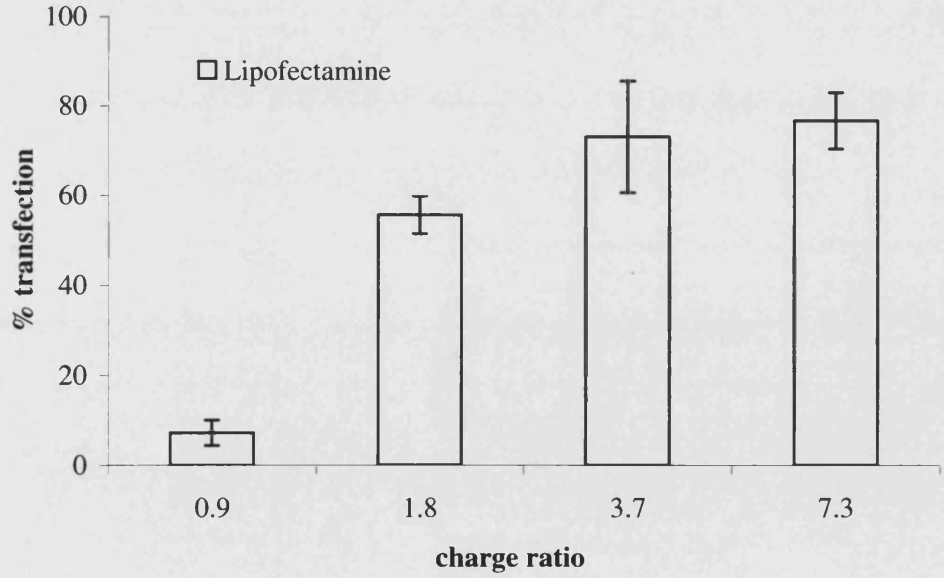


Figure 20. Lipofection of FEK4 cells transfected with pEGFP complexed with Lipofectamine at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

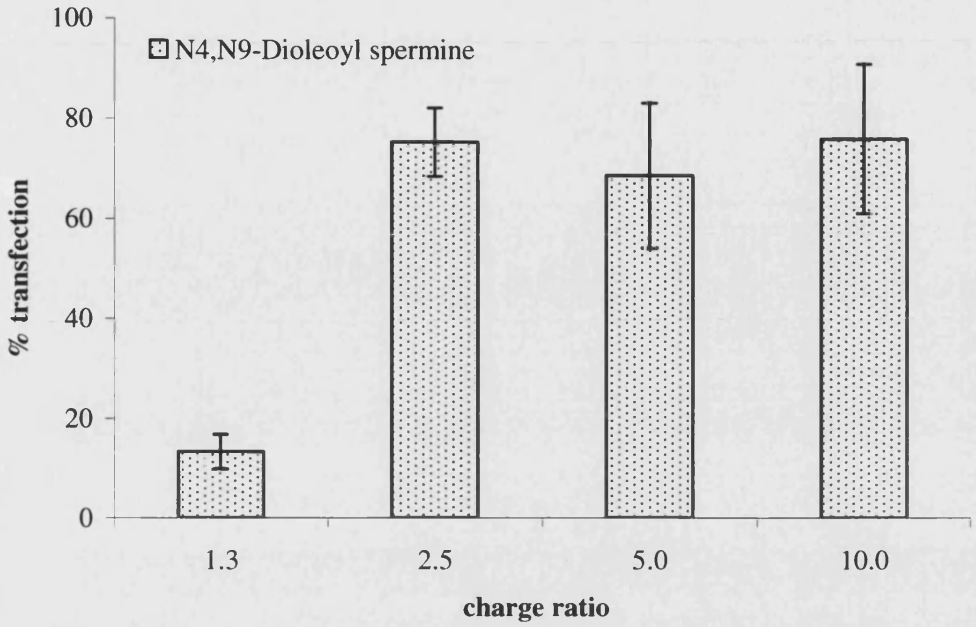


Figure 21. Lipofection of HtTA cells transfected with pEGFP complexed with N^4,N^9-dioleoyl spermine at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

To compare the transfection results of the lipoplexes in both the primary and cancer cell lines, Fig. 22 show the comparison of the expression levels in both FEK4 and HtTA cells. Expression levels results of Lipofectin-pEGFP lipoplex in HtTA were higher than the transfection level achieved by the lipoplex in the primary cell line FEK4 at the same charge ratio investigated. It is shown in the literature that the cancer cell line are generally transfected easily compared with the primary cell lines (238, 239).

On the other hand, Lipofectamine and N^4,N^9-dioleoyl spermine lipoplexes with pEGFP transfection results show almost no significant improvement in the expression levels of green fluorescent protein in HtTA cells in comparison with the results achieved in FEK4 cells (Fig. 22). This could be attributed to the high efficiency of Lipofectamine and N^4,N^9-dioleoyl spermine in transfecting the primary cell line FEK4, transfection efficiency levels in the case of FEK4 were about 70 % and 75 % in the case of Lipofectamine and N^4,N^9-dioleoyl spermine, respectively.

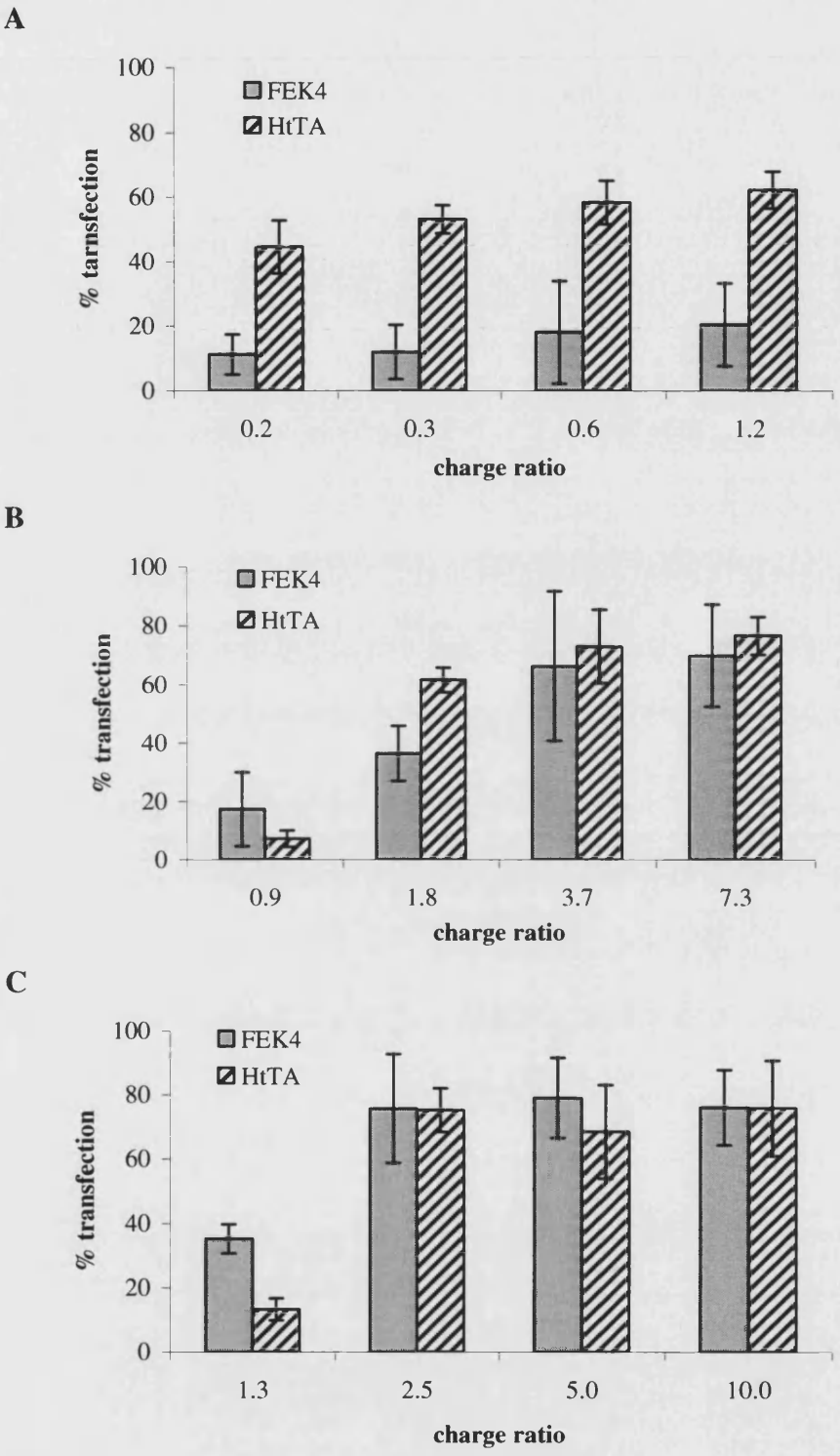


Figure 22. Comparison of the transfection efficiency at different charge ratios of FEK4 and HtTA cells transfected with pEGFP complexed with **A** Lipofectin, **B** Lipofectamine or **C** N^4,N^9-dioleoyl spermine. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

In-vitro cytotoxicity

Mosmann (240) has developed a rapid colorimetric assay that is widely used for analysing the cytotoxic effect of the gene delivering agents. The living cells when incubated with (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide produced a dark blue insoluble formazan crystal that is solubilzed in dimethylsulfoxide (DMSO) and measured spectrophotometrically (570 nm) as shown in Fig. 23.

The cytotoxicity of N^4,N^9-dioleoyl spermine and the liposomal formulations Lipofectin and Lipofectamine were studied in FEK4 and HtTA cells. The IC_{50} values (the concentration at which cell growth is inhibited by 50 % (113)) for the investigated lipopolyamines were calculated. Mikos and his research group reported (228) that the free PEI is more toxic than the DNA complexed form. So, the cytotoxicity studies were carried out on both the free and complexed form to investigate if there is a significant difference in the toxic effects (IC_{50}) of the free cationic lipid over the lipoplex form in the case of HtTA cells and FEK4 cells.

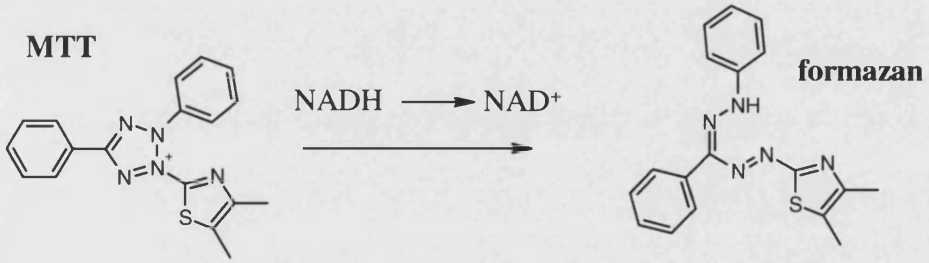


Figure 23. MTT and the reaction product formazan.

The cytotoxicity of Lipofectin, as free or complexed with pEGFP, was studied in FEK4 and HtTA cells as shown in Fig. 24. The IC_{50} values for the free polycation in FEK4 and HtTA cells were 11 µg/ml, and for the lipoplex were 9.5 and 6.5 µg/ml respectively. The results indicate that there is no significant difference in the toxic effect (IC_{50}) of the free polycation over the lipoplex in the case of HtTA cells and FEK4 cells for the studied lipopolyamine.

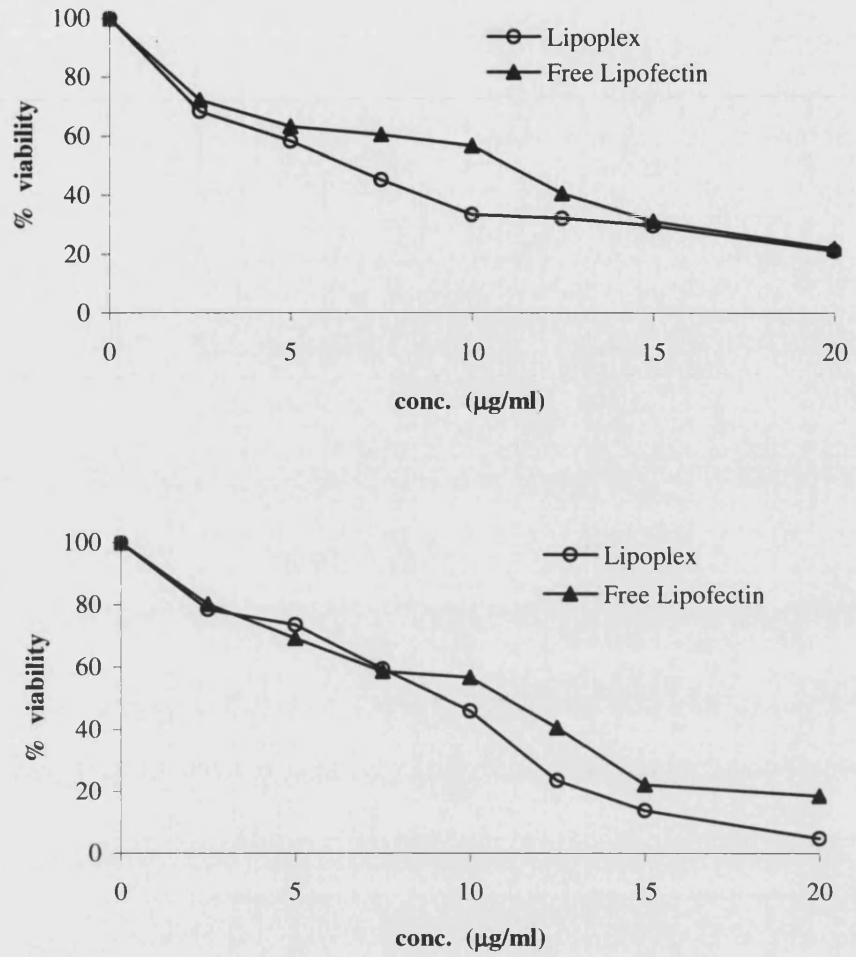
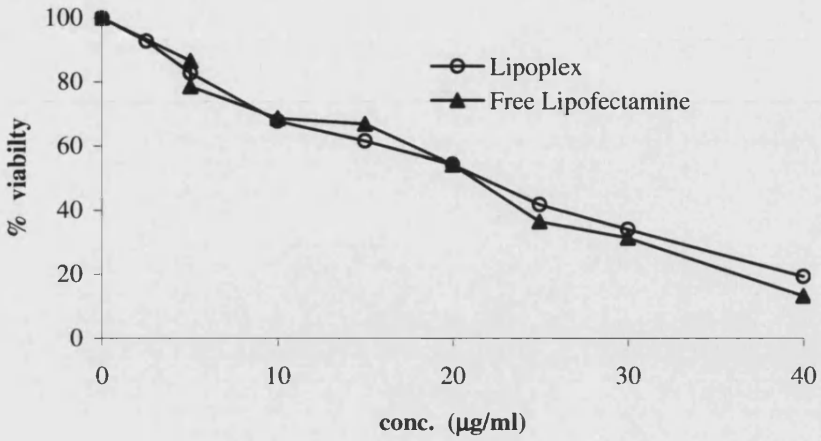


Figure 24. Viability of HtTA cells (above) and FEK4 primary skin cells (below) after application of different concentrations of Lipofectin either free or complexed with pEGFP.

In the case of Lipofectamine, the cytotoxicity as free or complexed with pEGFP, was studied in FEK4 and HtTA cells are shown in Fig. 25. The IC_{50} values for the free cationic lipid in FEK4 and HtTA cells were 19 and 21 µg/ml respectively, and for the lipoplex were 25 and 21 µg/ml respectively. The results indicate that there is no significant difference in the toxic effect (IC_{50}) of the free polycation over the lipoplex in the case of HtTA cells and FEK4 cells for the studied lipopolyamine.



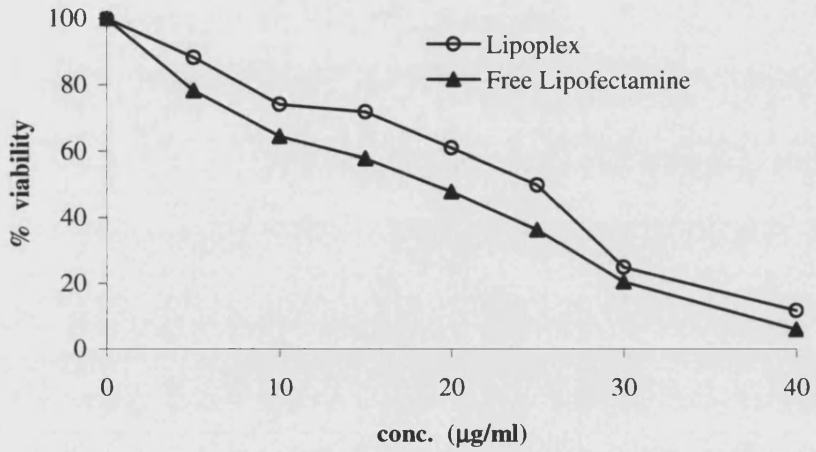


Figure 25. Viability of HtTA cells (above) and FEK4 primary skin cells (below) after application of different concentrations of Lipofectamine either free or complexed with pEGFP.

In the case of N^4,N^9-dioleoyl spermine, the IC_{50} values for the free lipopolyamine in FEK4 and HtTA cells were 20.47 and 6.31 µg/ml respectively, and for the lipoplex were 19.82 and 8.35 µg/ml respectively (Fig. 26). The results indicate that there is no significant difference in the toxic effect (IC_{50}) of the free polycation over the lipoplex in the case of HtTA cells and FEK4 cells for the studied lipopolyamine. The results show that cell viability levels in the case of FEK4 are higher than in HtTA cells. These results also revealed that N^4,N^9-dioleoyl spermine toxicity is higher (lower concentrations) in the case of HtTA cells more than with the primary cell line FEK4 which could be attributed to the ease of transfection of immortalized cancer cell lines over primary cell lines.

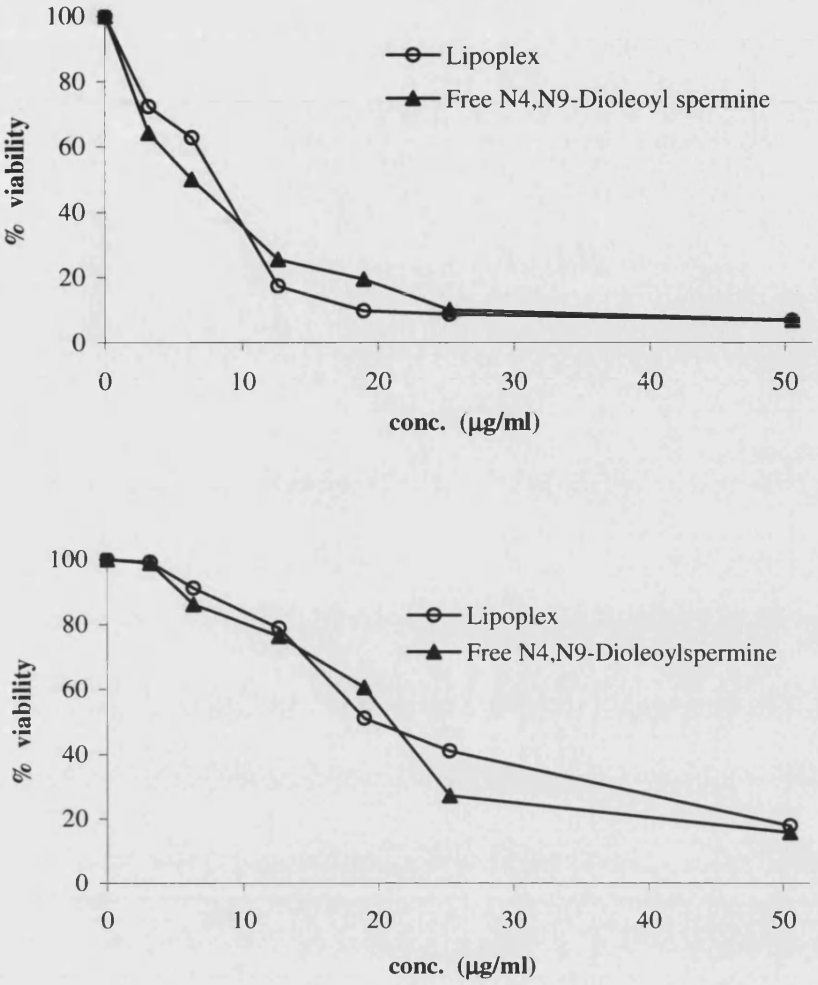


Figure 26. Viability of HtTA cells (above) and FEK4 primary skin cells (below) after application of different concentrations of N^4,N^9-dioleoyl spermine either free or complexed with pEGFP.

Figs. 27 and 28 show the transfection efficiency results in relation to cell viability levels at different concentrations of Lipofectin, Lipofectamine and N^4,N^9-dioleoyl spermine for both the primary cell line FEK4 and the cancer cell line HtTA. These figures were plotted in order to determine the optimum charge ratio (concentration) of transfection. The optimum charge ratio of transfection is selected based on the best transfection results a cationic lipid can achieve in relation to acceptable cytotoxic levels at the same charge ratio. The results in Figs. 27 and 28 show that the optimum charge ratios selected are 0.6, 3.7 and 2.5 for Lipofectin, Lipofectamine and N^4,N^9-dioleoyl spermine, respectively. Although the cationic lipids achieve higher transfection results at higher charge ratios, but this accompanied with increase in the toxicity levels of the cationic lipid.

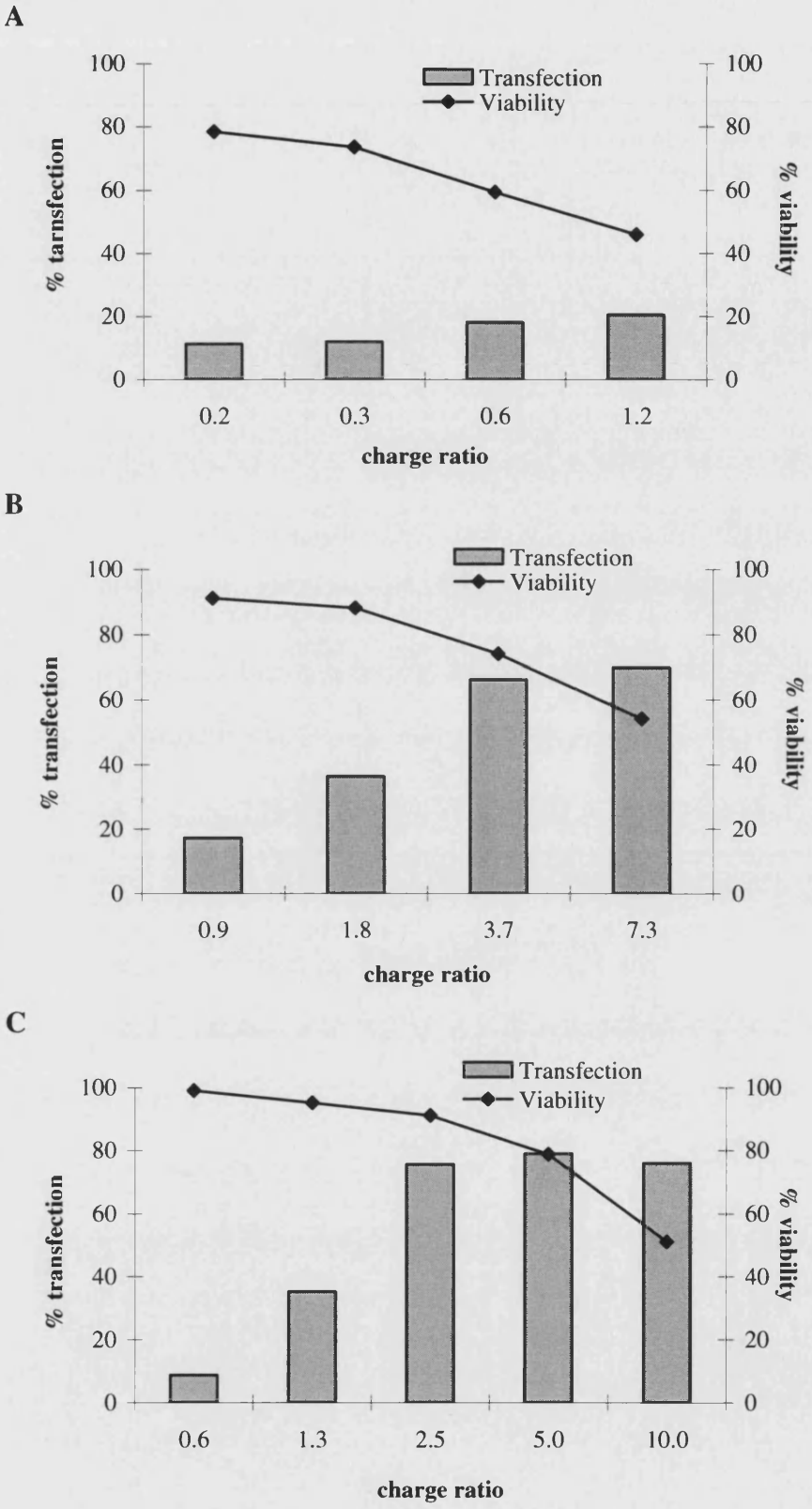
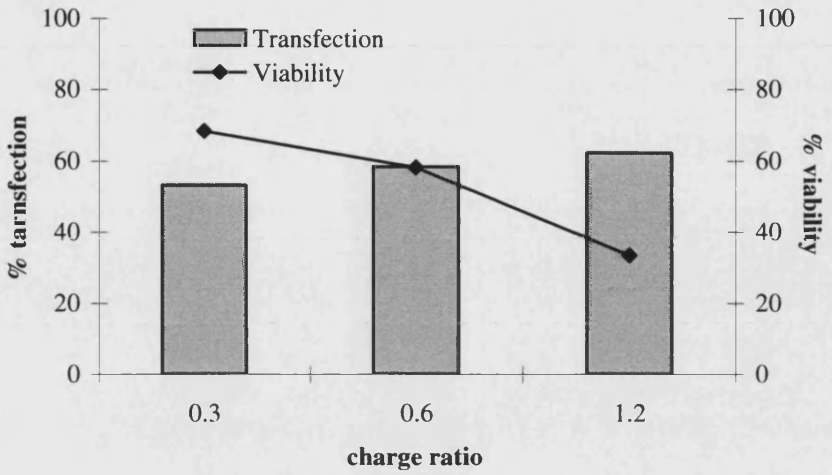
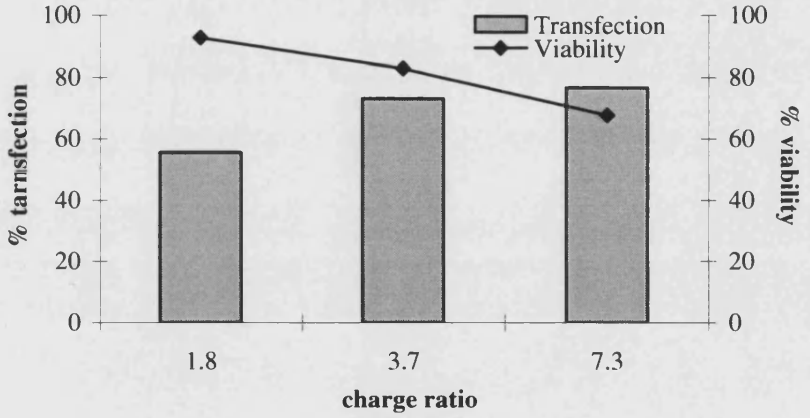


Figure 27. Lipofection and cytotoxicity studies of FEK4 cells transfected with pEGFP complexed with **A** Lipofectin®, **B** Lipofectamine™ or **C** N^4,N^9-dioleoyl spermine at different N/P charge ratios.

A



B



C

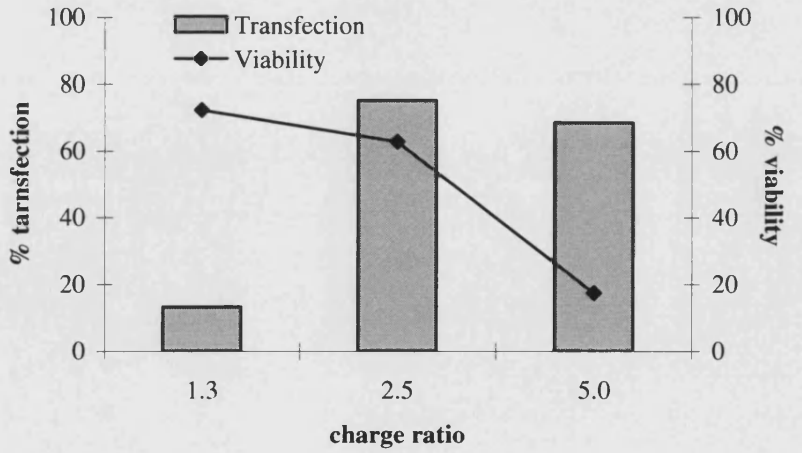


Figure 28. Lipofection and cytotoxicity studies of HtTA cells transfected with pEGFP complexed with **A** Lipofectin®, **B** Lipofectamine™ or **C** N^4,N^9-dioleoyl spermine at different N/P charge ratios.

Fig. 29 shows the cytotoxic effect of the investigated cationic lipids at their optimum charge ratio of transfection. From these results it was found that, N^4,N^9-dioleoyl spermine show a significant difference in the % viability comparing to either Lipofectin® and Lipofectamine in the primary skin cell line FEK4, but there is no difference in the case of the cancer cell line HtTA (Fig. 10). These results also revealed that N^4,N^9-dioleoyl spermine toxicity is higher (at lower concentrations) in the case of HtTA cells more than with the primary cell line FEK4 which could be attributed to the ease of transfection of immortalized cancer cell lines over primary cell lines, as a result of higher uptake rates of the plasmid (239).

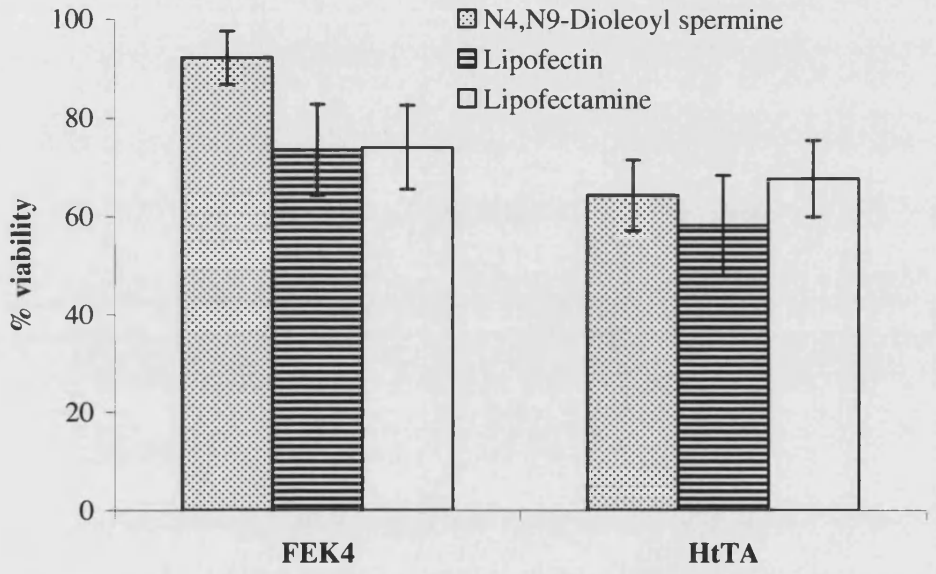


Figure 29. Cytotoxicity effect of pEGFP (2 μg/ml) complexed with either N^4,N^9-dioleoyl spermine (5.54 μg/ml), Lipofectin® (5 μg/ml) or Lipofectamine™ (10 μg/ml) in FEK4 and HtTA cells.

Also, the transfection efficiency of the cationic lipids at their optimum charge ratio of transfection were compared with the transfection results of "naked" DNA, as negative control, as indicated in Fig. 30. The transfection results of pEGFP into FEK4 indicated higher transfection ability of N^4,N^9-dioleoyl spermine (75 %) and Lipofectamine™ (66 %) formulations over Lipofectin® (18 %). On the other hand, there is no significant difference in the transfection activity between N^4,N^9-dioleoyl spermine and Lipofectamine™ (Fig. 30).

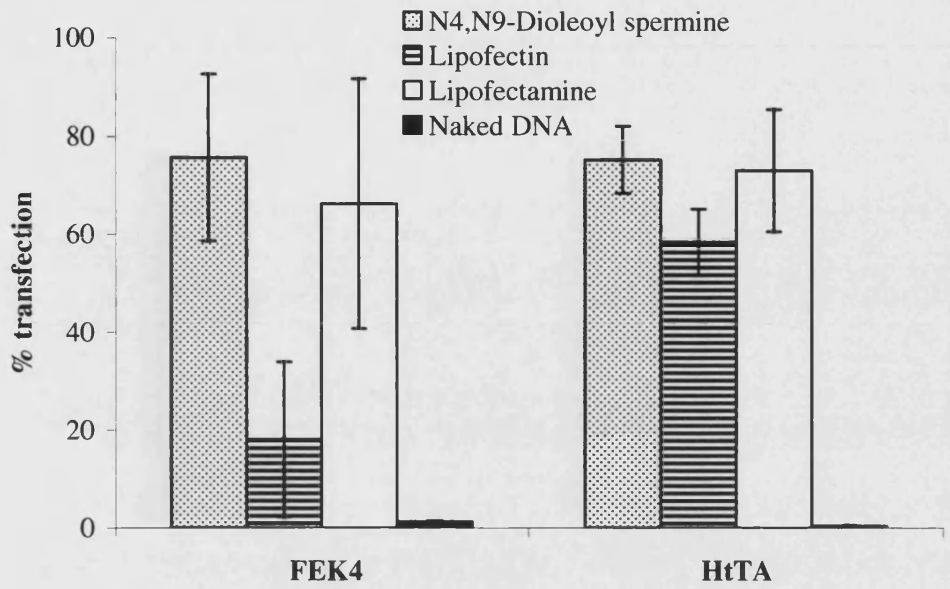


Figure 30. Lipofection of FEK4 and HtTA cells transfected with pEGFP either free (naked) or complexed with Lipofectin®, Lipofectamine™ or N^4,N^9-dioleoyl spermine (at their respective N/P ratio for best transfection). The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

While in the case of HtTA transfection results, N^4,N^9-dioleoyl spermine and Lipofectamine™ formulations show a similar transfection activity (about 70 %), higher than Lipofectin® (58 %) (Fig. 30). N^4,N^9-Dioleoyl spermine transfects the cells best at charge ratio (+/-) 2.5 (5.54 µg/ml), Lipofectin® at charge ratio 0.6 (5.0 µg/ml), while Lipofectamine™ transfect both cell lines at charge ratio 3.7 (10.0 µg/ml).

These results revealed that the difficulties found in efficiently transfecting primary cell lines were largely overcome with the non-liposomal formulation N^4,N^9-dioleoyl spermine comprising a vector with two covalently bound oleoyl chains. The helper lipid DOPE which is the second component of the cationic liposomal formulations is used to increase the transfection activity of the cationic liposomes through its ability to destabilise lipid bilayer leading to endosomal destabilisation with subsequent increase in the total cellular uptake of the delivered DNA (91, 209).

Lipospermines (Fig. 2) with their cationic head-group sometimes form micelles (in the absence of DNA) rather than the bilayer produced by the small cationic quaternary ammonium head-group of DOTMA (Lipofectin® formulation) (226, 241). N^4,N^9-Dioleoyl spermine combines in its structure the two oleoyl chains that have the characteristics of the fusogenic lipid DOPE and the cationic polyamine spermine (Fig. 2). Thus, DNA is condensed by the two primary amines and coated by the dioleoyl lipophilic moiety. The DNA condensation results obtained with N^4,N^9-dioleoyl spermine (Fig. 10) show a higher efficiency than either liposomal formulation.

The transfection results reveal higher transfection ability (levels of expression) of N^4,N^9-dioleoyl spermine compared to Lipofectin® (Fig. 30) in FEK4 and HtTA cells. These results indicate the roles of both number and distribution of positive charges along the polyamine backbone on the ability of the compound to condense DNA. On the other hand, there is no significant difference in the transfection activity between N^4,N^9-dioleoyl spermine and Lipofectamine™ in both FEK4 and HtTA cell lines, which indicates the importance of lipid coating over the DNA molecule on both the condensation and cellular delivery of DNA in the case of the liposomal and non-liposomal formulations (241). N^4,N^9-Dioleoyl spermine achieved high levels of transfection in both a cancer cell line and a primary skin cell line (73 %) which indicates the ability of this vector to deliver DNA.

Cell viability results revealed improved FEK4 viability more than the cancer cells HtTA (Fig. 29). Also, N^4,N^9-dioleoyl spermine showed a significant improvement in primary FEK4 cell viability over the liposomal formulations (Fig. 29), but no significant difference in HtTA cells. In conclusion, in this chapter, a lipopolyamine vector has been developed for DNA delivery. This new non-liposomal formulation has the ability to transfect primary skin cells more efficiently than the commercially available liposomal Lipofectin® formulation. These promising results draw our attention to the role of the degree of unsaturation of the lipospermine lipid moiety in the transfection efficiency and therefore whether a change in the lipid hydrophobic domain could improve the interactions of the lipospermine with DNA and/or with cellular components (e.g. for endosome escape). In chapter 4, we investigate changes in the shape of the C18 lipid moieties.

CHAPTER 4

Efficient Non-Viral Gene Delivery Using C18-Lipid Conjugates of Spermine

Introduction

As viral gene therapy continues to suffer significant problems with mammalian toxicity (7, 8, 242), the possibility of reaching the goal of intracellular protein levels at therapeutic concentrations moves even more towards utilising non-viral gene therapy (NVGT). Within this broad term, we are concentrating on lipopolyamines composed either of a lipophilic steroid attached to a polyamine chain (145, 171, 210, 243, 244), or to a single or double long-carbon chain covalently bound to a polyamine e.g. spermine (N^1,N^{12}-diamino-4,9-diazadodecane) (241, 245). Other research groups are investing in a variety of alternative approaches classified under the NVGT umbrella, including: naked DNA (246), gene gun (bound to gold particles) (115, 247), electroporation (248), polycation-mediated DNA delivery, and the use of a wide variety of cationic lipids (lipoplexes) (63), e.g. bolaamphiphiles (69), and cationic polymers (polyplexes) (206, 249, 250), for reviews see: (10, 30, 251-253).

For gene therapy to realise its potential and become an efficient medicine for the treatment of diseases such as cancer, cystic fibrosis, inflammation, or for vaccination, key obstacles must be overcome. The essential requirements for gene delivery are the transport of DNA through the cell membrane and ultimately to the nucleus. The design of an efficient formula for the delivery of genetic material requires a detailed understanding of the barriers that hinder this process.

Thus, efficient formulations, lipoplexes (63, 69) polyplexes (206, 249, 250), must be able to deliver safely the required DNA across the various cellular barriers to the nucleus. Barriers to DNA delivery also include complex formation between the DNA and the lipopolyamine leading to DNA nanoparticle formation by electrostatic charge neutralisation (to about 90 % of the total negative charge) and overall packing as condensed DNA nanoparticles (22). So, a key first step in this method of gene formulation is masking the negative charges of the phosphate backbone. This titration with a lipopolyamine causes alleviation of charge repulsion between remote phosphates along the DNA helix leading to collapse into a more compact structure that facilitates cell entry.

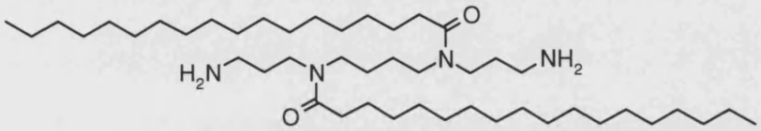
For NVGT, except for naked (free, uncomplexed) DNA being trapped inside cells by direct association with the chromatin during mitosis, the (prodrug) DNA must be formulated. Small molecule synthetic cationic lipids are one of the major gene carriers for NVGT, often classified as liposomal and non-liposomal non-viral delivery vectors. They condense DNA into nanoparticles that are readily endocytosed by cultured cells, and facilitate endosomal escape leading to efficient delivery to the nucleus presumably crossing through the nuclear pore complex. After nuclear entry, the payload DNA should ultimately be able to give the desired protein through transcription and translation.

The first cellular barrier for the delivery of the DNA nanoparticles is the eukaryotic cell membrane which is composed mainly of phospholipids (50-90 % of total lipid content, most phospholipids are derivatives of diacyl-glycerol-3-phosphate), sterols (5-25 %), and glycol-lipids (usually less than 5 %). The diacyl-glycerol chains (C14 to C24) are derived from linear fatty acids with varying degrees of unsaturation (254). In NVGT, cationic lipids which interact with the DNA payload also mediate cell-membrane transport, typically through adsorptive endocytosis or mediated by cations (157, 158, 255), both routes leading to internalization of the DNA complex nanoparticles. Such endocytosis is via the clathrin-coated pits (250-300 nm) (256), followed by fusion of the early endosome and sorting to the late endosomal compartment, hence avoiding degradation in the lysosome.

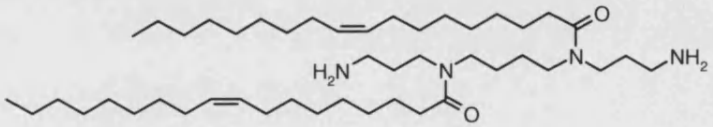
One of the important factors to improve the release of free DNA or the lipoplex into the cytoplasm is the influence of the cationic lipid chain. Xu and Szoka (93) have reported that unsaturated hydrocarbon chains increase the transfection efficiency of the lipoplex by decreasing the rigidity of the bilayer and favouring a higher inter-membrane transfer rate and lipid mixing, compared to their saturated counterparts. However, Engberts, Hoekstra, and co-workers recently reported that, in certain cases, saturated fatty chains afforded better results than unsaturated chains (257). Also, Heyes et al. (176) showed that, among the cationic lipid analogues they synthesized with variation in the degree of saturation, the saturated C18 conjugates were internalized in to the cells more, although they are less efficient in gene silencing (siRNA delivery).

In this chapter, we investigate the effects on DNA formulation of a circular plasmid with variation in the degree of unsaturation in the two C18 fatty chains in our lipospermines, the saturated N^4,N^9-distearoyl spermine, the alkenoic N^4,N^9-dioleoyl spermine, and the dienoic fatty acyl spermine conjugate N^4,N^9-dilinoleoyl spermine (Fig. 1). We report the synthesis of the lipopolyamines and characterisation of the lipoplexes nanoparticles formed, transfection results with the synthesized lipospermine formulations in a panel of both primary and cancer cell lines, and compare these results with those obtained with the commercially available non-liposomal lipospermine Transfectam® (dioctadecylamidoglycylspermine, DOGS) formulation (179, 185).

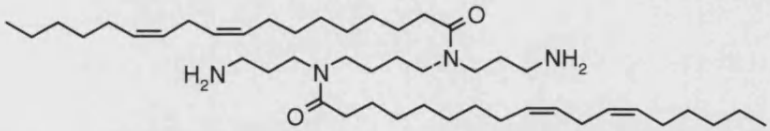
N^4,N^9-Distearoyl spermine



N^4,N^9-Dioleoyl spermine



N^4,N^9-Dilinoleoyl spermine



Transfectam® (DOGS)

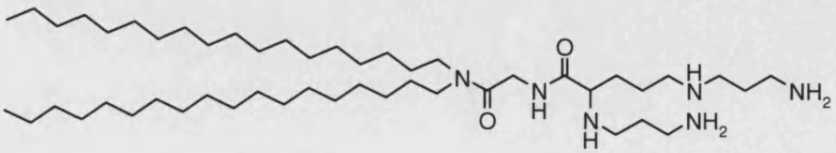


Figure 1. Spermine based lipopolyamines, N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine, N^4,N^9-dilinoleoyl spermine and Transfectam®.

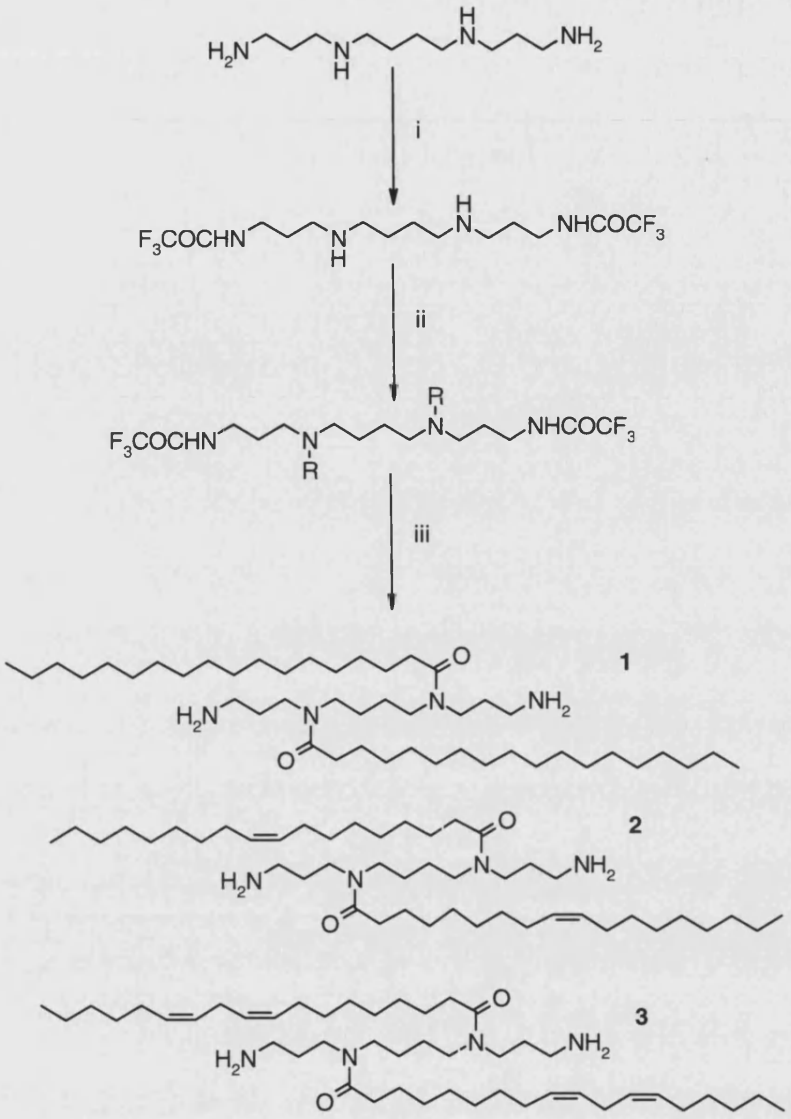


Figure 2. Synthetic scheme of spermine based cationic lipids: N^4,N^9-distearoyl spermine **1**, N^4,N^9-dioleoyl spermine **2**, and N^4,N^9-dilinoleoyl spermine **3**. Reagents: i) ethyl trifluoroacetate, methanol, 18 h at 20 °C; ii) fatty acyl chloride, R = (stearoyl **1**, oleoyl **2**, and linoleoyl **3**), triethylamine, CH_2Cl_2 and methanol (1:1), 72 h at 20 °C; iii) methanol saturated with ammonia gas, 18 h at 20 °C.

Synthesis of lipospermines N^4,N^9-dilinoleoyl spermine, N^4,N^9-dioleoyl spermine, and N^4,N^9-distearoyl spermine

The tetra-amine spermine was used as the starting material for the synthesis of the three desired lipopolyamines: N^4,N^9-distearoyl spermine **1**, N^4,N^9-dioleoyl spermine **2**, N^4,N^9-dilinoleoyl spermine **3,** outlined in Fig. 2. The tetra-amine was protected on both the primary amino functional groups with ethyl trifluoroacetate (2.2 eq.) in methanol and the reaction mixture was stirred for 18 h at 20 °C to form N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane R_f 0.7 (CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v).

Each corresponding fatty acyl chloride (stearoyl, oleoyl, or linoleoyl) (2.2 eq) was used as the acylating agent together with triethylamine (2.5 eq) to the diprotected spermine solution in CH_2Cl_2 and methanol (1:1). The solution was stirred for 72 h at 20 °C, and then evaporated to dryness in vacuo. The residue was dissolved in CH_2Cl_2 and the solution filtered and evaporated to dryness in vacuo to form N^4,N^9-(dilinoleoyl, dioleoyl (187), or distearoyl)-N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane.

For the removal of the di-trifluoroacetyl protecting groups, the tetra-amide was dissolved in methanol and the pH of the solution was increased by saturating with ammonia gas, then it was left for 18 h at 20 °C, and then evaporated to dryness in vacuo to give a residue which was purified over silica gel (CH_2Cl_2-MeOH 5:3 v/v, then CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v) to afford the three desired lipopolyamine conjugates as their free bases. Flash column chromatography afforded the three target lipospermines, homogenous on silica gel thin-layer chromatography and characterized by high-resolution mass spectroscopy (HRMS).

N^4,N^9-Distearoyl spermine **1** R_f = 0.41 (CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v), $C_{46}H_{95}N_4O_2$ found (m/z $[M+H]^+$) 735.7458, requires 735.7455 (Δ ppm -0.4), $C_{45}{}^{13}CH_{95}N_4O_2$ found 736.7500, requires 736.7489 (Δ ppm -1.5), $C_{46}H_{95}{}^{2}HN_4O_2$ found 736.7500, requires 736.7533 (Δ ppm 4.6). N^4,N^9-Dioleoyl spermine **2** R_f = 0.44 (CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v), $C_{46}H_{91}N_4O_2$ found (m/z $[M+H]^+$)

731.7115, requires 731.7142 (Δ ppm 3.7), $C_{45}{}^{13}CH_{91}N_4O_2$ found 732.7145, requires 732.7176 (Δ ppm 4.1). N^4,N^9-Dilinoleoyl spermine **3** R_f = 0.49 (CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v), $C_{46}H_{87}N_4O_2$ found (m/z $[M+H]^+$) 727.6846, requires 727.6829 (Δ ppm -2.3), $C_{45}{}^{13}CH_{87}N_4O_2$ found 728.6857, requires 728.6863 (Δ ppm 0.7).

Preparation of plasmid DNA

We have chosen to deliver a 4.7 kbp plasmid encoding for enhanced green fluorescent protein (pEGFP), with a molecular weight of about 3.1 MegaDa (given an average of 330 Da per nucleotide, 660 Da per bp (5), carrying 9,400 negative charges. DNA plasmid pEGFP, purchased from Clontech, was transformed into *Escherichia coli* JM 109 bacterial strain (Promega) and purified by a Qiagen Maxi kit (Qiagen). DNA yields and purity were determined spectroscopically (OD_{260}/OD_{280} = 1.80–1.90 OD, optical density) and by agarose gel (1 %) analysis.

DNA condensation

In this study, we have investigated the change in the degree of unsaturation of the di-C18 fatty chain formulation of lipospermine on DNA condensation and formation of nanoparticles. DNA condensation was monitored using an ethidium bromide (EthBr) fluorescence quenching and light scattering assays as described in detail in the materials and methods chapter. The results were compared with the commercially available lipospermine Transfectam®.

Ethidium bromide fluorescence quenching assay

DNA (6 μg) was diluted to 3 ml with buffer (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) in a glass cuvette stirred with a micro-flea. EthBr solution (3 μl, 0.5 mg/ml) was added to the stirring solution and allowed to equilibrate for 10 min. Separately, lipopolyamine aliquots (5 μl, according to the ammonium/phosphate (+/-) charge ratio required) were then added to the stirring solution and the fluorescence measured after 1 min equilibration using a Perkin-Elmer LS 50B luminescent

spectrometer (λ_{excit} = 260 nm and λ_{emiss} = 600 nm with slit width 5 nm) while stirring using an electronic stirrer (Rank Bros. Ltd.). The fluorescence was expressed as the percentage of the maximum fluorescence when EthBr was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free EthBr in solution.

Behr, Vierling and co-workers (185) showed that the commercially available non-liposomal formulation Transfectam® (DOGS, Fig. 1) has a pK_a values of 10.5, 9.5, 8.4 and 5.5, and according to Henderson-Hasselbach equation, Transfectam® has 3 positive charges / molecule (more accurately 2.92) at pH 7.4. They also indicate that the lowest nitrogen has the same pK_a value of the lysosome pH (5.5) which will have a role to improve the transfection efficiency of the lipospermine through buffering the decrease in the pH inside the endosome.

Fig. 3 shows the DNA condensation ability of the synthesized lipopolyamine formulations in comparison with the commercially available Transfectam® in an EthBr fluorescence quenching assay. Three cationic lipid formulations N^4,N^9-dilinoleoyl spermine, N^4,N^9-dioleoyl spermine, and the commercially available Transfectam® show similar DNA condensing ability. Also, they are able to condense DNA completely (defined as more than 90 % EthBr fluorescence quenching (19, 22)) at N/P charge ratio 2, while N^4,N^9-distearoyl spermine is only able to displace around 80 % of EthBr at the same charge ratio.

To quantify the ability of the investigated lipopolyamines in condensing DNA, the binding constant of these polyamines with DNA was estimated (Table 1) and compared using EthBr assay that has been shown to be a valid method for comparison of DNA binding affinity (144, 194, 221). The drug concentration producing 50 % inhibition of fluorescence is approximately inversely proportional to the binding constant (194). From Table 1 data, the concentrations of condensing agents producing 50 % fluorescence inhibition were calculated as shown in the experimental chapter. The K_{app} of lipopolyamines (N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine, N^4,N^9-dilinoleoyl spermine and Transfectam®) binding was calculated taking into account that the binding constant of ethidium is 10^7 M^{-1} (194) and the concentration of EthBr is 1.3 μM. From these results it was found that

Transfectam® has the highest binding affinity (K_{app} $1.1x10^7$ M^{-1}) between the compared polyamines and N^4,N^9-distearoyl spermine the lowest with binding affinity (K_{app} $0.4x10^7$ M^{-1}) which could be attributed to the higher number of positive charges (+3.0) and the charge distribution on Transfectam® compared with N^4,N^9-distearoyl spermine that has two positive charges.

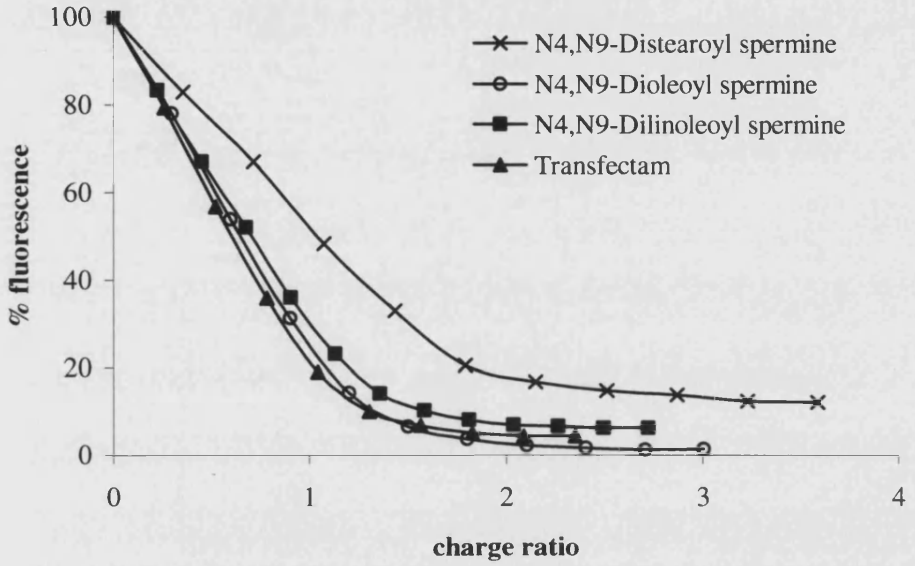


Figure 3. Plot of EthBr fluorescence quenching assay of calf thymus DNA complexed with N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine, N^4,N^9-dilinoleoyl spermine, or Transfectam®.

Table 1. Calf thymus DNA binding data

Condensing agent	Drug concentration at 50 % fluorescence quenching (μM)	K_{app*}
N^4,N^9-distearoyl spermine	3.3	$4x10^6$ M^{-1}
N^4,N^9-dioleoyl spermine	1.7	$8x10^6$ M^{-1}
N^4,N^9-dilinoleoyl spermine	1.8	$7x10^6$ M^{-1}
Transfectam®	1.2	$1.1x10^7$ M^{-1}

* "apparent" equilibrium binding constant of condensing agent to calf thymus DNA

Light scattering assay

The apparent UV absorbance at 320 nm showing light scattering was measured to investigate the condensation of DNA by lipopolyamines and the formation of particles (20). The formation of DNA nanoparticles causes scattering of light that reduces light intensity at the UV detector.

Fig. 4 shows the increase in apparent absorbance by light scattering of the formed particles as a result of interaction of N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine and N^4,N^9-dilinoleoyl spermine with calf thymus DNA. The maximum apparent absorption was reached at a charge ratio of 2.5, 1.7 and 1.5 in the case of N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine and N^4,N^9-dilinoleoyl spermine, respectively. These results indicate improved ability of N^4,N^9-dioleoyl spermine and N^4,N^9-dilinoleoyl spermine to condense DNA and form particles over N^4,N^9-distearoyl spermine, which were also similar with the results concluded from EthBr fluorescence quenching study (Fig. 3).

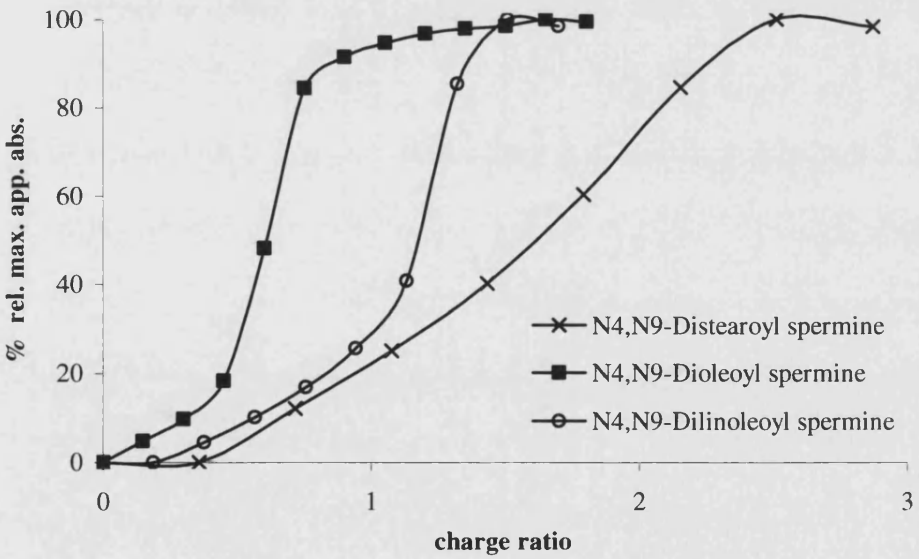


Figure 4. Light scattering assay (relative maximum apparent absorbance at $\lambda = 320$ nm) of calf thymus DNA with the lipospermines: N^4,N^9-distearoyl spermine N^4,N^9-dioleoyl spermine and N^4,N^9-dilinoleoyl spermine.

Fig. 5 shows the plot of EthBr fluorescence quenching and light scattering data of N^4,N^9-dilinoleoyl spermine interacted with calf thymus DNA. These results reveal that the light scattering due to particle formation increases with the increase in the displaced EthBr and reaches the maximum at approximately the same charge ratio region (1.5-1.7) at which there is a maximum EthBr displacement. This comparison show that the validity of both experiments to investigate DNA condensation studies, although the concentration of DNA used in light scattering experiments is ten times the concentration used in fluorescence quenching experiments which is related to the lack of sensitivity of light scattering experiment in comparison with EthBr fluorescence assay.

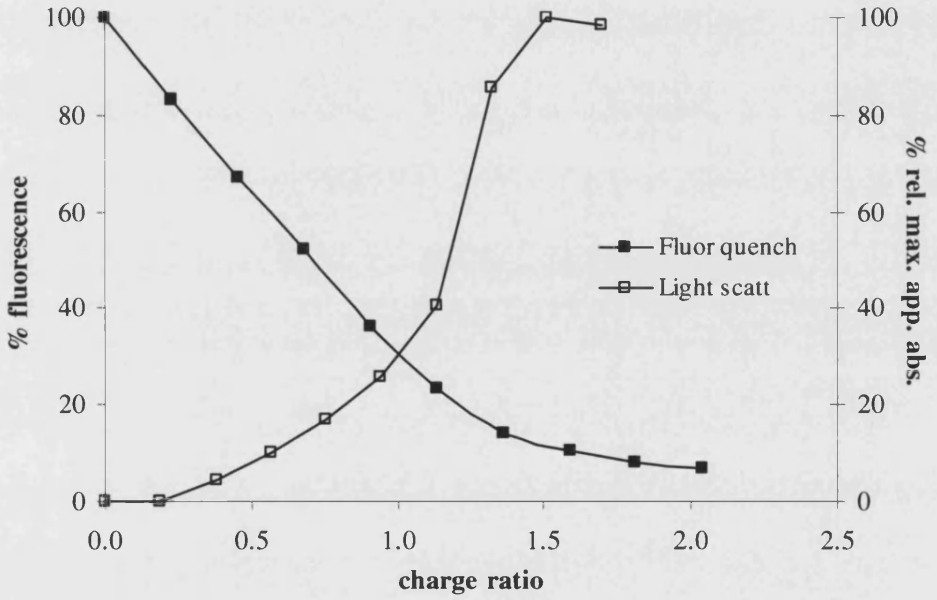


Figure 5. Comparison of EthBr fluorescence quenching and light scattering assays of calf thymus DNA with N^4,N^9-dilinoleoyl spermine.

Cell culture and transfection experiments

Six cell lines were used in the transfection experiment, FEK4 (196), FCP4, FCP5, FCP7 and FCP8 cells are human primary fibroblasts derived from newborn foreskin explants (198). HtTA cells are a human cervical carcinoma, HeLa derived and transformed cell line. Cells were cultured in Earle's Minimal Essential Medium (EMEM) supplemented with foetal calf serum (FCS) 15 % in the case of FEK4 and FCP cells and 10 % in the case of HtTA cells, penicillin and streptomycin (50 IU/ml

each), glutamine (2 mM), and sodium bicarbonate (0.2 %). Primary cells were passaged once a week, and used between passages 7-15.

For the transfection (gene delivery) and the resultant gene activity (transfection efficiency), cells were seeded in 6-well plates at a density of 1 x 10^5 cells/well (in 4 ml EMEM with FCS) for 24 h to reach a 50-60 % confluency on the day of transfection. The complex was prepared by mixing pEGFP (2 μg/ml/well) with each lipopolyamine in Opti-MEM (serum free media, Gibco BRL) according to the charge ratio at 20 °C for 30 mins, and then incubated with the cells for 4 h at 37 °C in 5 % CO_2. Then the cells were washed and cultured for a further 44 h in growth medium at 37 °C in 5 % CO_2.

Levels of EGFP in the transfected cells were detected and corrected for background fluorescence of the control cells using a fluorescence activated cell sorting (FACS) machine (Becton Dickinson FACS Vantage dual Laser Instrument, argon ion laser 488 nm). The transfection efficiency was calculated based on the percentage of the cells that expressed EGFP (positive cells) in the total number of cells.

Primary skin cell transfection studies

A panel of five primary skin cell lines were used in the transfection experiment, FEK4, FCP4, FCP5, FCP7 and FCP8 cells are human primary fibroblasts derived from newborn foreskin explants. The use of a group of primary cell lines is to validate the transfection efficiency results of the investigated lipopolyamines as primary cell lines are well known as a difficult to transfect cell lines (233, 234).

The synthesized lipopolyamines and Transfectam® were investigated for their gene delivery ability in FCP4 cells using pEGFP as a reporter gene. Fig. 6 shows the transfection results of pEGFP into FCP4 using N^4,N^9-distearoyl spermine at different charge ratios. These results show that N^4,N^9-distearoyl spermine is unable to achieve high transfection efficiency in FCP4 cell line up to charge ratio 20. Fig. 6 shows that N^4,N^9-distearoyl spermine achieves a maximum of 21 % transfection efficiency from charge ratio 20. In the case of Transfectam®, results shown in Fig. 7 revealed that

commercially available lipopolyamine achieves higher transfection efficiency compared to N^4,N^9-distearoyl spermine. Transfectam® shows the highest gene expression levels of about 80 % at charge ratio 9.8.

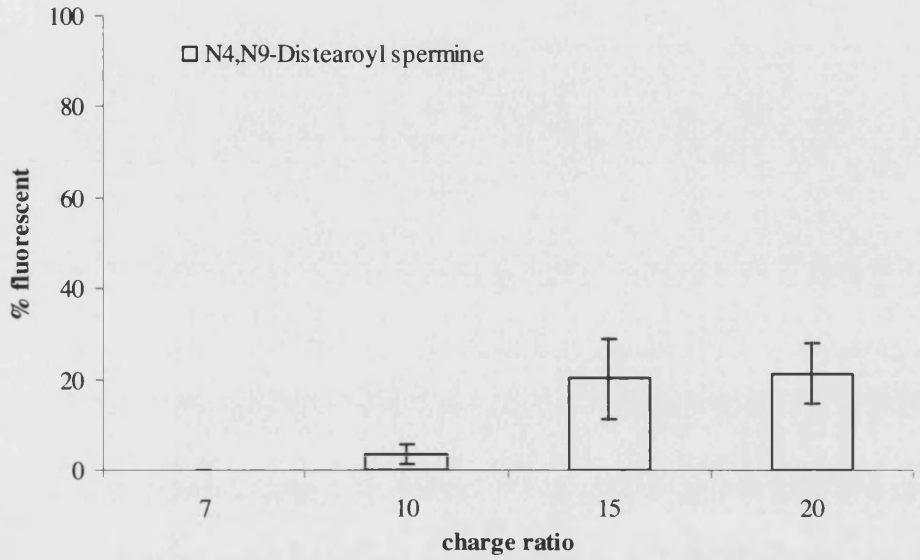


Figure 6. Lipofection of FCP4 cells transfected with pEGFP complexed with N^4,N^9-distearoyl spermine at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

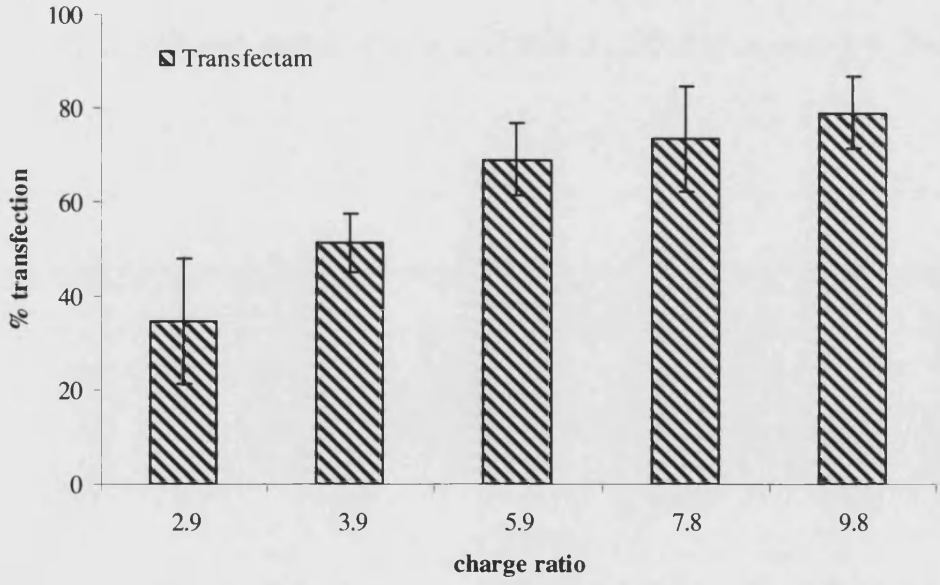


Figure 7. Lipofection of FCP4 cells transfected with pEGFP complexed with Transfectam® at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

On the other hand, N^4,N^9-dilinoleoyl spermine show higher transfection efficiency levels in comparison with N^4,N^9-distearoyl spermine, Transfectam® and N^4,N^9-dioleoyl spermine that achieves 62 % transfection efficiency at the optimum charge ratio of transfection (N/P 2.5). N^4,N^9-Dilinoleoyl spermine achieves transfection levels more than 80 % from charge ratio 5.5, as indicated in Fig. 8.

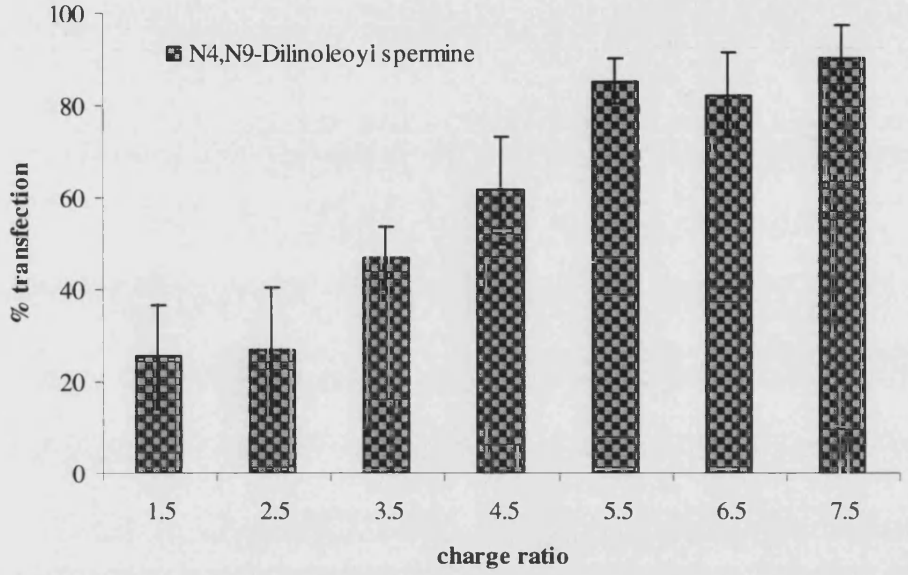


Figure 8. Lipofection of FCP4 cells transfected with pEGFP complexed with N^4,N^9-dilinoleoyl spermine at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

In the case of FCP5 cell line, transfection results of N^4,N^9-distearoyl spermine show that the lipopolyamine was only able to achieve 31 % of transfection efficiency at charge ratio 15 (Fig. 9). Transfectam® was able to achieve higher green fluorescent protein expression levels of more than 60 % from charge ratio 5.9 as shown in Fig. 10. Similarly, N^4,N^9-dioleoyl spermine achieves 68 % transfection efficiency at charge ratio 2.5. On the other hand, N^4,N^9-dilinoleoyl spermine shows a significant improvement in the transfection efficiency over the investigated lipopolyamines. The double unsaturated C18 spermine conjugate achieves more than 90 % transfection efficiency from charge ratio 5.5 as indicated in Fig. 11.

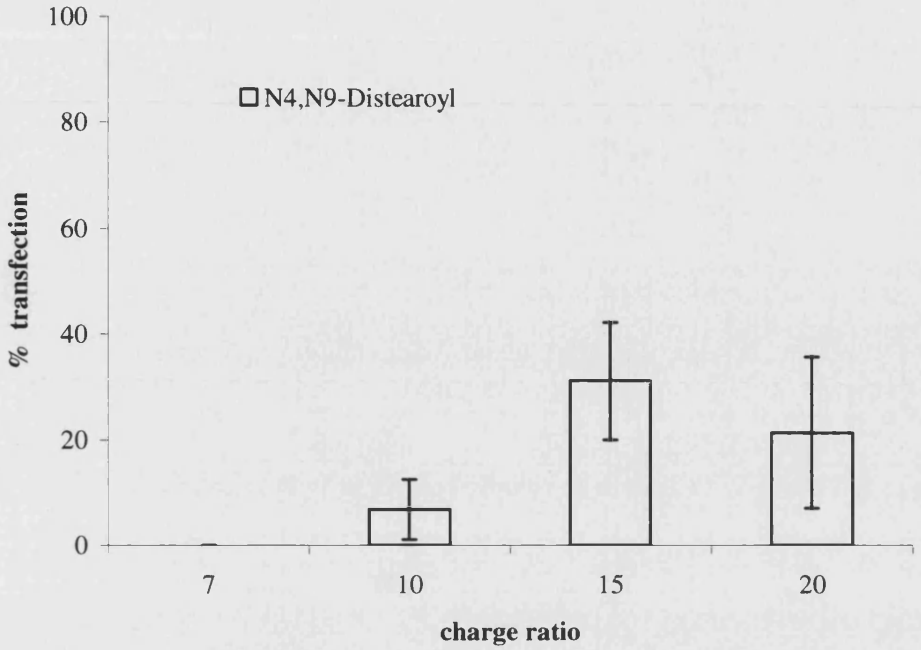


Figure 9. Lipofection of FCP5 cells transfected with pEGFP complexed with N^4,N^9-distearoyl spermine at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

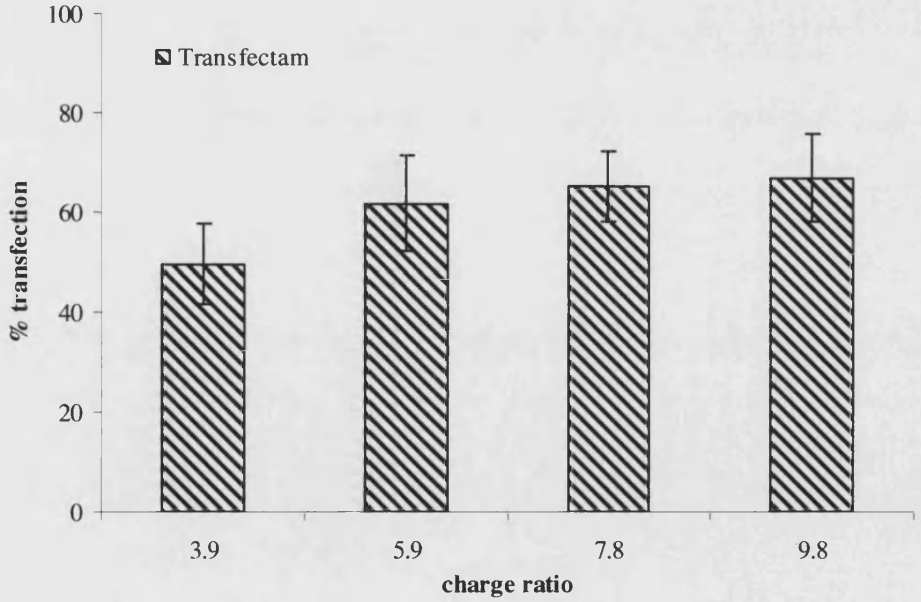


Figure 10. Lipofection of FCP5 cells transfected with pEGFP complexed with Transfectam® at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

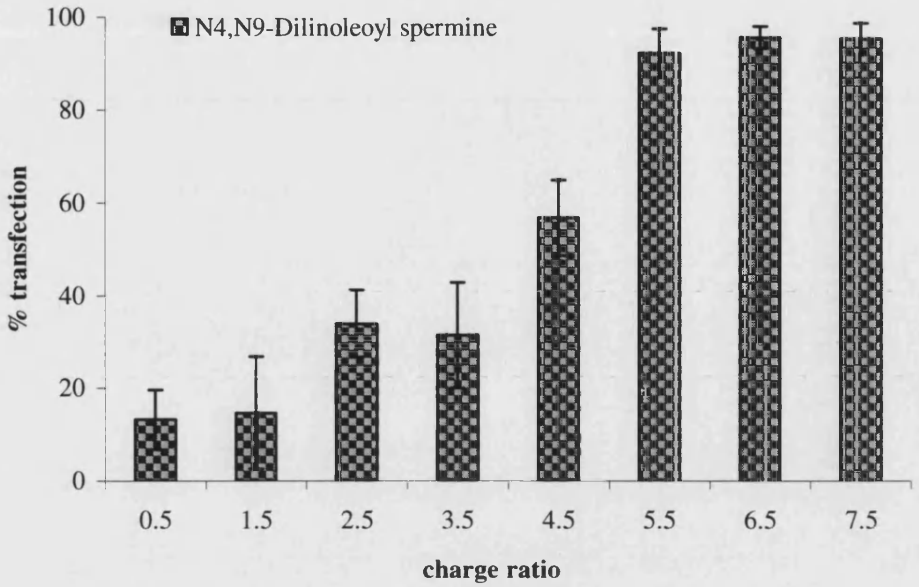


Figure 11. Lipofection of FCP5 cells transfected with pEGFP complexed with N^4,N^9-dilinoleoyl spermine at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

In the case of FCP7 cell line, N^4,N^9-distearoyl spermine is not able to transfect the cells efficiently. The results in Fig. 12 show that N^4,N^9-distearoyl spermine is able to transfect FCP7 cell line by 24 % at charge ratio 15. Also, the transfection results show that the lipopolyamine is not able to transfect the cell line at charge ratios lower than N/P 10. While in the case of Transfectam®, the results revealed that the commercially available lipopolyamine achieves higher transfection levels by more than 54 % from charge ratio 5.9, as shown in Fig. 13. N^4,N^9-Dioleoyl spermine transfects the cell line by 61 % at charge ratio 2.5. Also similar to FCP5 cells transfection results, N^4,N^9-dilinoleoyl spermine continues to show a significant improvement in the transfection levels Fig. 14. The cationic lipid achieves more than 90 % transfection efficiency from charge ratio 5.5 as shown in Fig. 14.

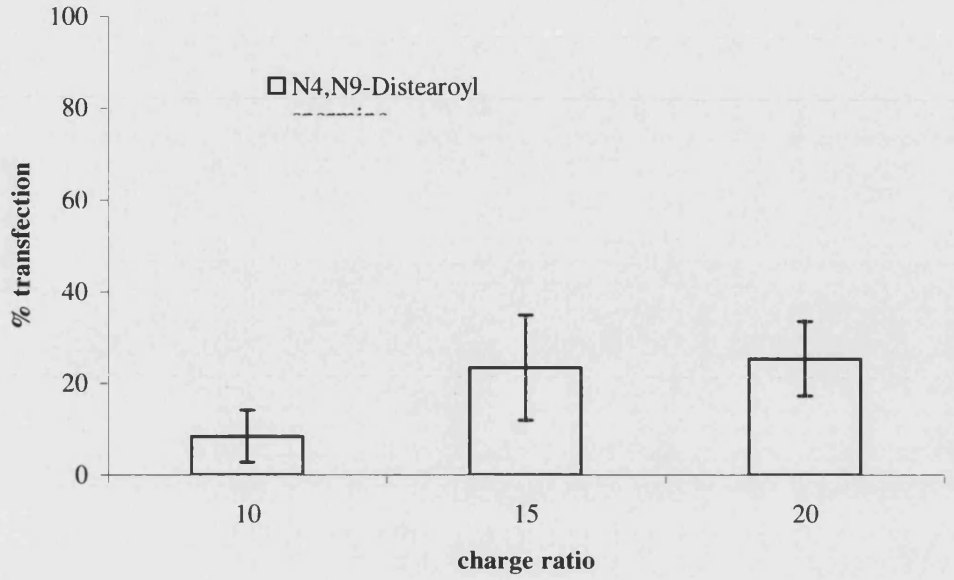


Figure 12. Lipofection of FCP7 cells transfected with pEGFP complexed with N^4,N^9-distearoyl spermine at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

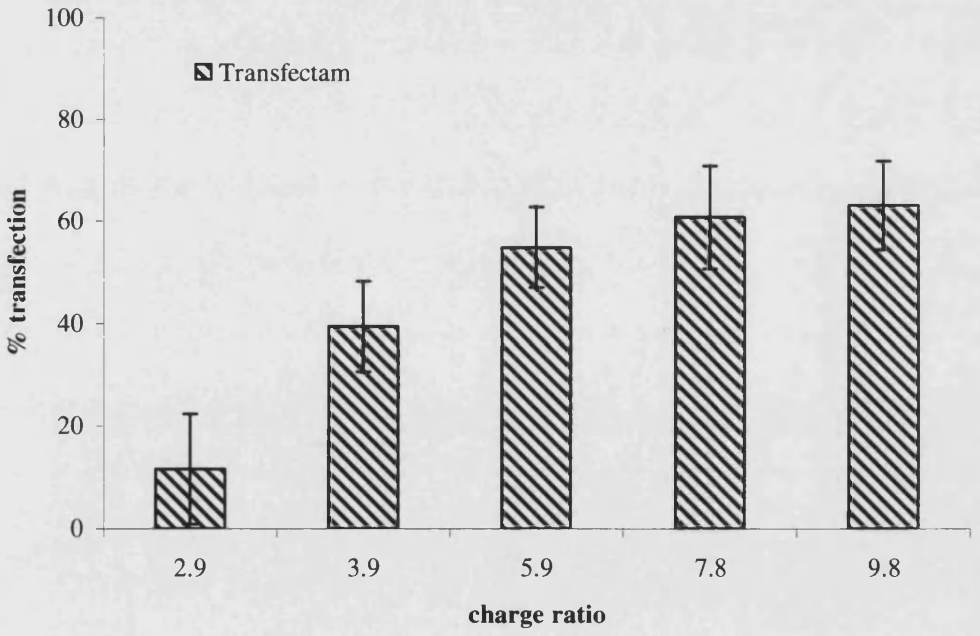


Figure 13. Lipofection of FCP7 cells transfected with pEGFP complexed with Transfectam® at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

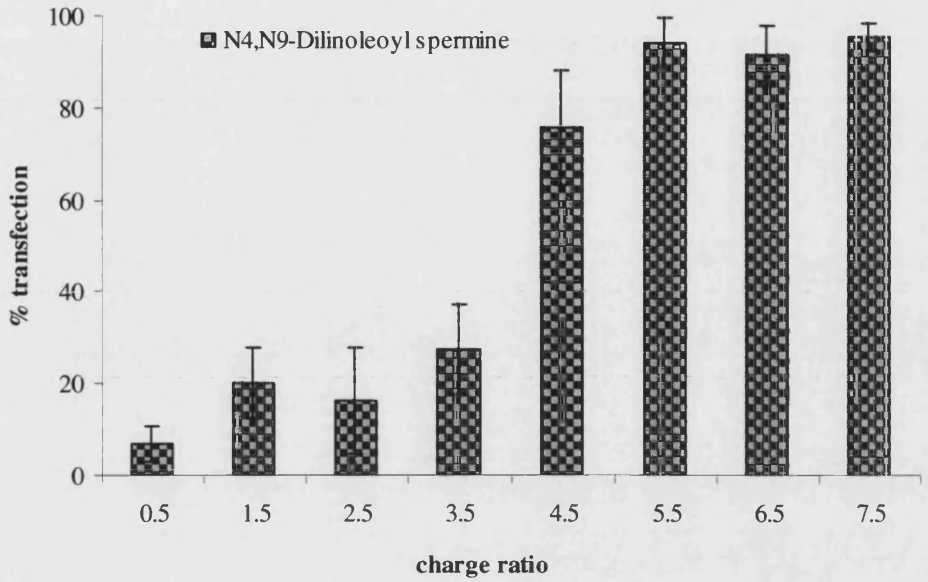


Figure 14. Lipofection of FCP7 cells transfected with pEGFP complexed with N^4,N^9-dilinoleoyl spermine at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

Transfection results of the primary skin cell line FCP8 show that N^4,N^9-distearoyl spermine is not able to transfect the cells efficiently. The results revealed in Fig. 15 show that N^4,N^9-distearoyl spermine is able to transfect FCP8 cell line by 22 % at charge ratio 20. While Transfectam® achieves higher transfection levels by up to 52 % at charge ratio 9.8 (Fig. 16). N^4,N^9-Dioleoyl spermine transfects the cell line by 65 % at charge ratio 2.5. The transfection results of pEGFP into FEK4 using N^4,N^9-dilinoleoyl spermine indicated a high transfection ability of N^4,N^9-dioleoyl spermine more than 75 % transfection efficiency from charge ratio 5.5 (Fig. 17). Also, there is no significant increase in the transfection ability of N^4,N^9-dilinoleoyl spermine-pEGFP lipoplex above charge ratio 5.5, as indicated in Fig. 17.

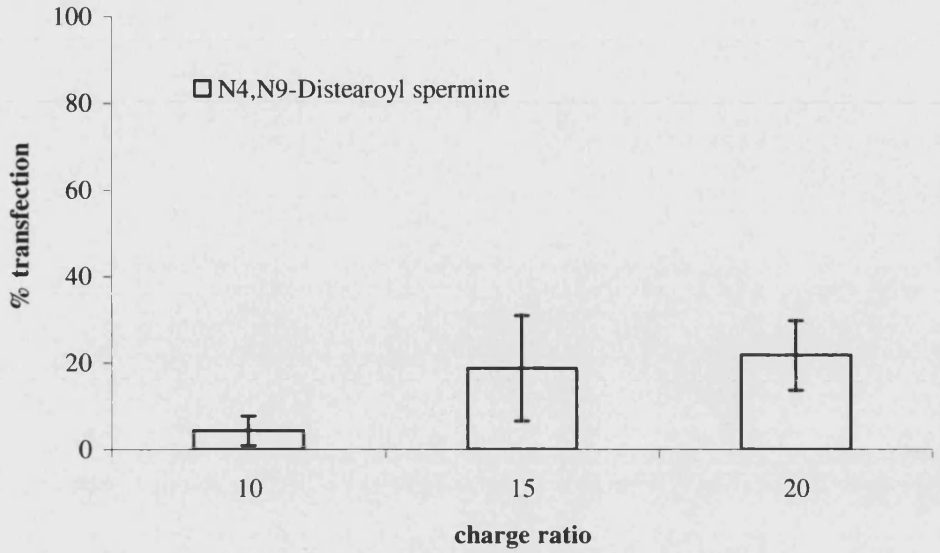


Figure 15. Lipofection of FCP8 cells transfected with pEGFP complexed with N^4,N^9-distearoyl spermine at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

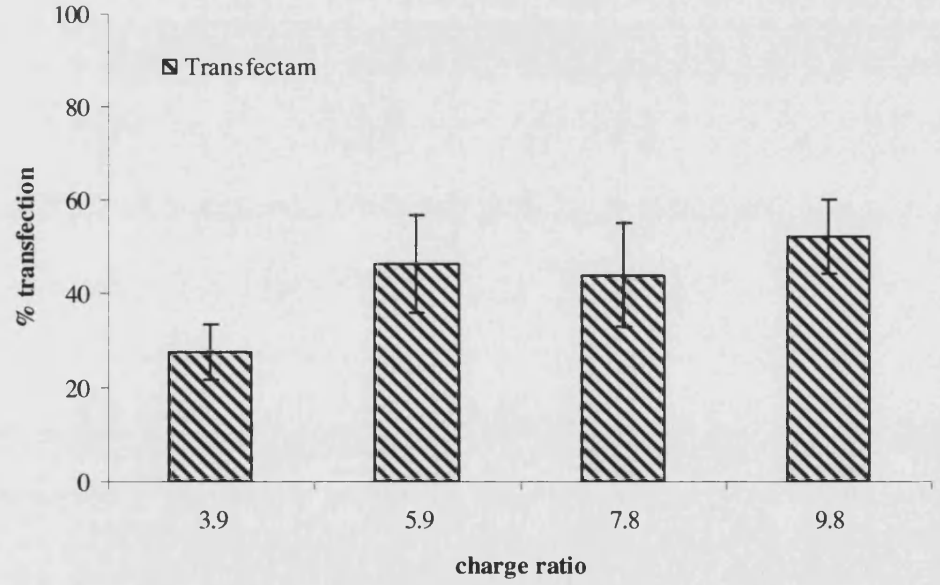


Figure 16. Lipofection of FCP8 cells transfected with pEGFP complexed with Transfectam® at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

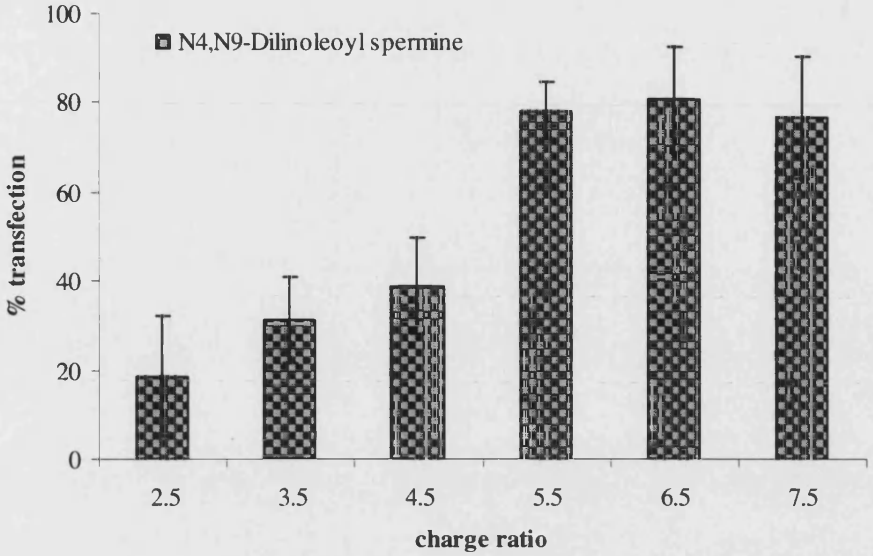


Figure 17. Lipofection of FCP8 cells transfected with pEGFP complexed with N^4,N^9-dilinoleoyl spermine at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

Fig. 18 shows the transfection results of pEGFP into FEK4 cells using N^4,N^9-distearoyl spermine at different charge ratios. Results show that N^4,N^9-distearoyl spermine achieves a maximum of only 30 % at charge ratio 20. The di-C18 saturated spermine conjugate is unable to deliver DNA to primary cell lines efficiently. On the other hand Transfectam®-DNA lipoplex shows a significant increase in the transfection efficiency by up to 79 % at charge ratio 5.9 (Fig. 19). As shown in the previous chapter, N^4,N^9-Dioleoyl spermine transfects the cell line by 76 % at charge ratio 2.5. In addition, N^4,N^9-dilinoleoyl spermine cationic lipid achieves more than 85 % transfection efficiency levels from charge ratio 5.5 as shown in Fig. 20. These results revealed that N^4,N^9-dilinoleoyl spermine is the most efficient cationic lipid compared to the investigated lipopolyamine for primary cell line transfection.

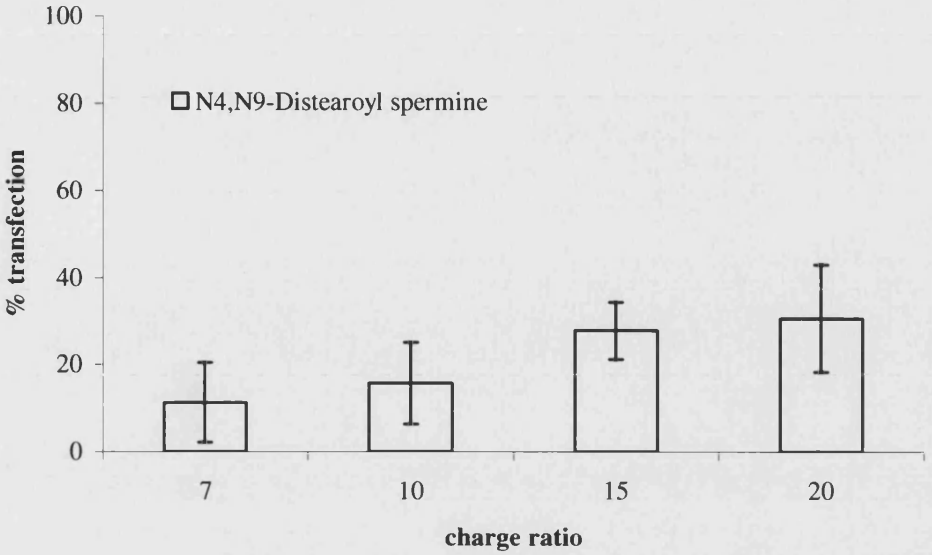


Figure 18. Lipofection of FEK4 cells transfected with pEGFP complexed with N^4,N^9-distearoyl spermine at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

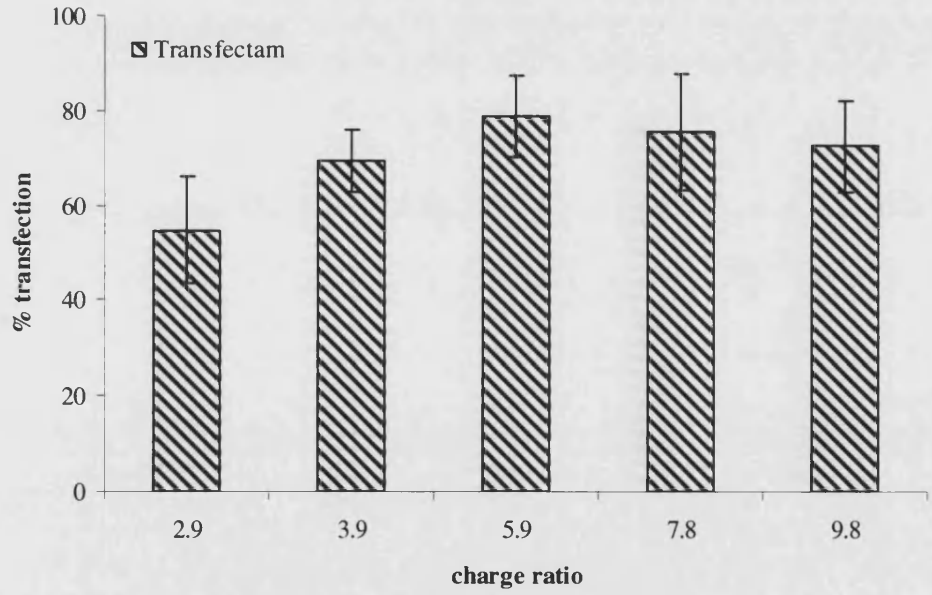


Figure 19. Lipofection of FEK4 cells transfected with pEGFP complexed with Transfectam® at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

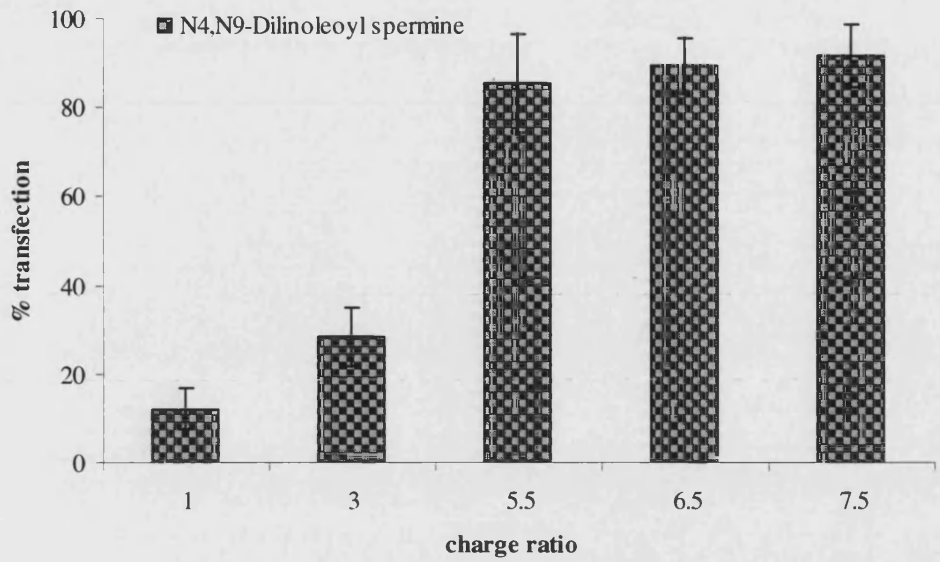


Figure 20. Lipofection of FEK4 cells transfected with pEGFP complexed with N^4,N^9-dilinoleoyl spermine at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

Cancer cell transfection studies

HeLa derivative and transformed cell line HtTA was used as a model for continuous cancer cell line to investigate the transfection ability of the studied lipopolyamines. Expression levels results of N^4,N^9-distearoyl spermine-pEGFP lipoplex in HtTA at different charge ratios show that the cationic lipid achieves a maximum of 26 % at charge ratio 15 (Fig. 21). The expression levels were improved by the use of the lipopolyamine Transfectam® lipoplex to 79 % (Fig. 22).

Transfectam® show a significant improvement in the transfection levels of the cancer cell line HtTA compared to the primary cell lines at different charge ratios, from 61 % at charge ratio 2.9 to up to 80 % transfection levels at charge ratio 9.8 (Fig. 22). N^4,N^9-Dioleoyl spermine achieves 75 % transfection efficiency at the optimum charge ratio of transfection 2.5 as indicated in the previous chapter. The lipofection N^4,N^9-dilinoleoyl spermine-pEGFP lipoplexes at different charge ratios show a significant increase in the transfection efficiency by increasing the charge ratio (Fig. 23) up to charge ratio 5.5 (99 %). There is no significant increase in the transfection efficiency at higher charge ratios than 5.5.

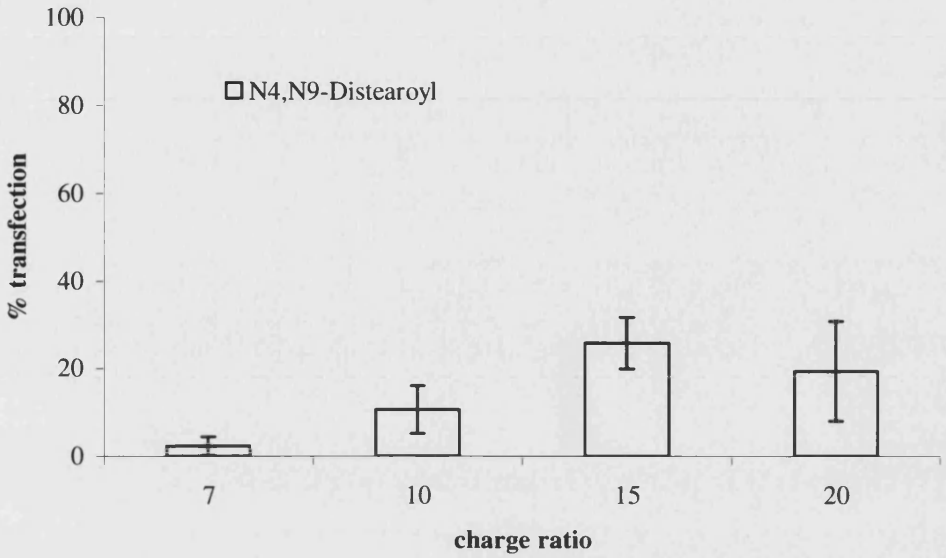


Figure 21. Lipofection of HtTA cells transfected with pEGFP complexed with N^4,N^9-distearoyl spermine at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

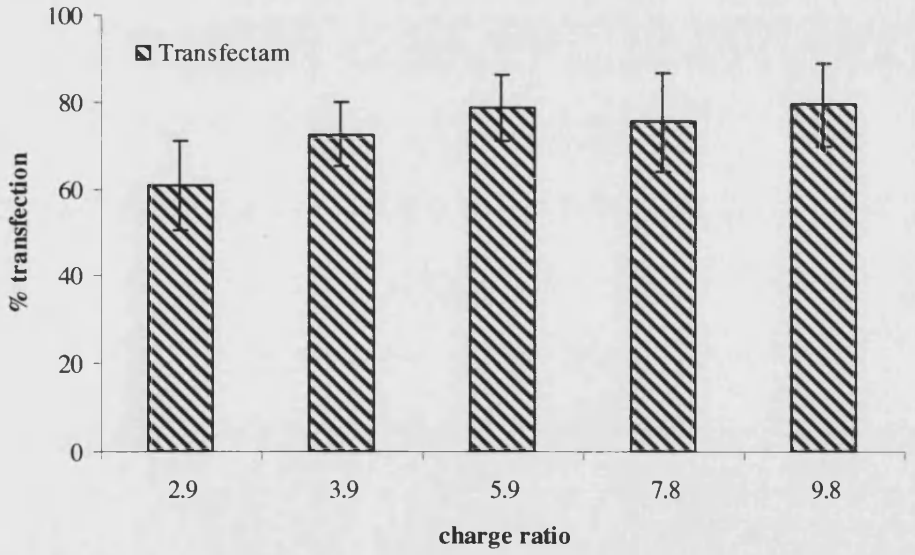


Figure 22. Lipofection of HtTA cells transfected with pEGFP complexed with Transfectam® at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

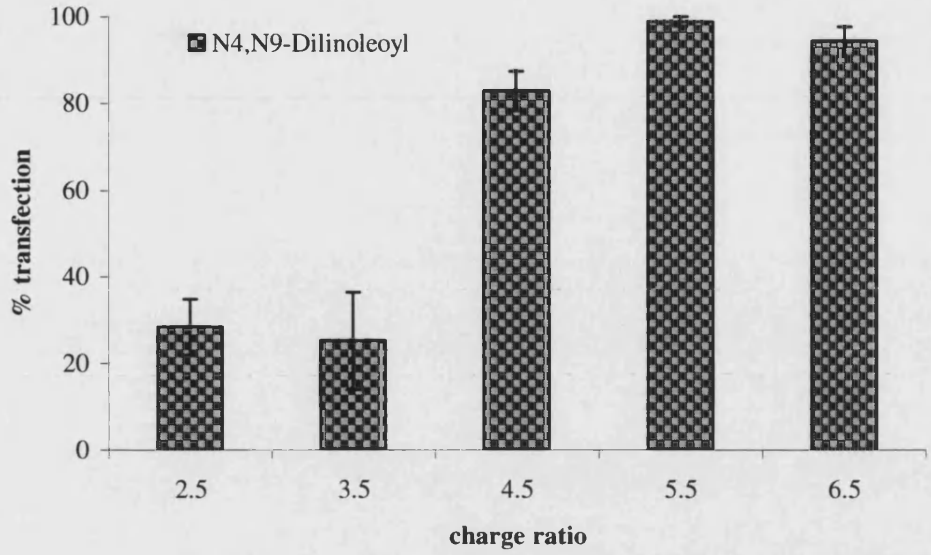


Figure 23. Lipofection of HtTA cells transfected with pEGFP complexed with N^4,N^9-dilinoleoyl spermine at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

In-vitro cytotoxicity

A panel of five primary skin cell lines FEK4, FCP4, FCP5, FCP7 and FCP8 cells and cancer cell line HtTA used in the transfection experiment were used for the cytotoxicity studies. The cytotoxicity of the synthesized lipopolyamines and the commercially available lipopolyamine Transfectam® was studied. The cytotoxicity of N^4,N^9-distearoyl spermine complexed with pEGFP was studied in the primary cell lines FCP4, FCP5 and FCP7 cells at different charge ratios as shown in Fig. 24. The lipoplex show high levels (more than 50 %) cell viability up to charge ratio 20 in the investigated cell lines. The results indicate 76 %, 63 % and 73 % cell viability at charge ratio 20 in the case of FCP4, FCP5 and FCP7, respectively. This could be related to the inability of the lipoplex to cross the cellular membrane, one of the main gene delivery barriers, which clear from the low transfection levels and higher viability levels.

In the case of the primary cell line FCP8 and FEK4 and the cancer cell line HtTA, N^4,N^9-distearoyl spermine lipoplex with pEGFP show high levels (more than 50 %) cell viability up to charge ratio 20 at different charge ratios (Fig. 25).

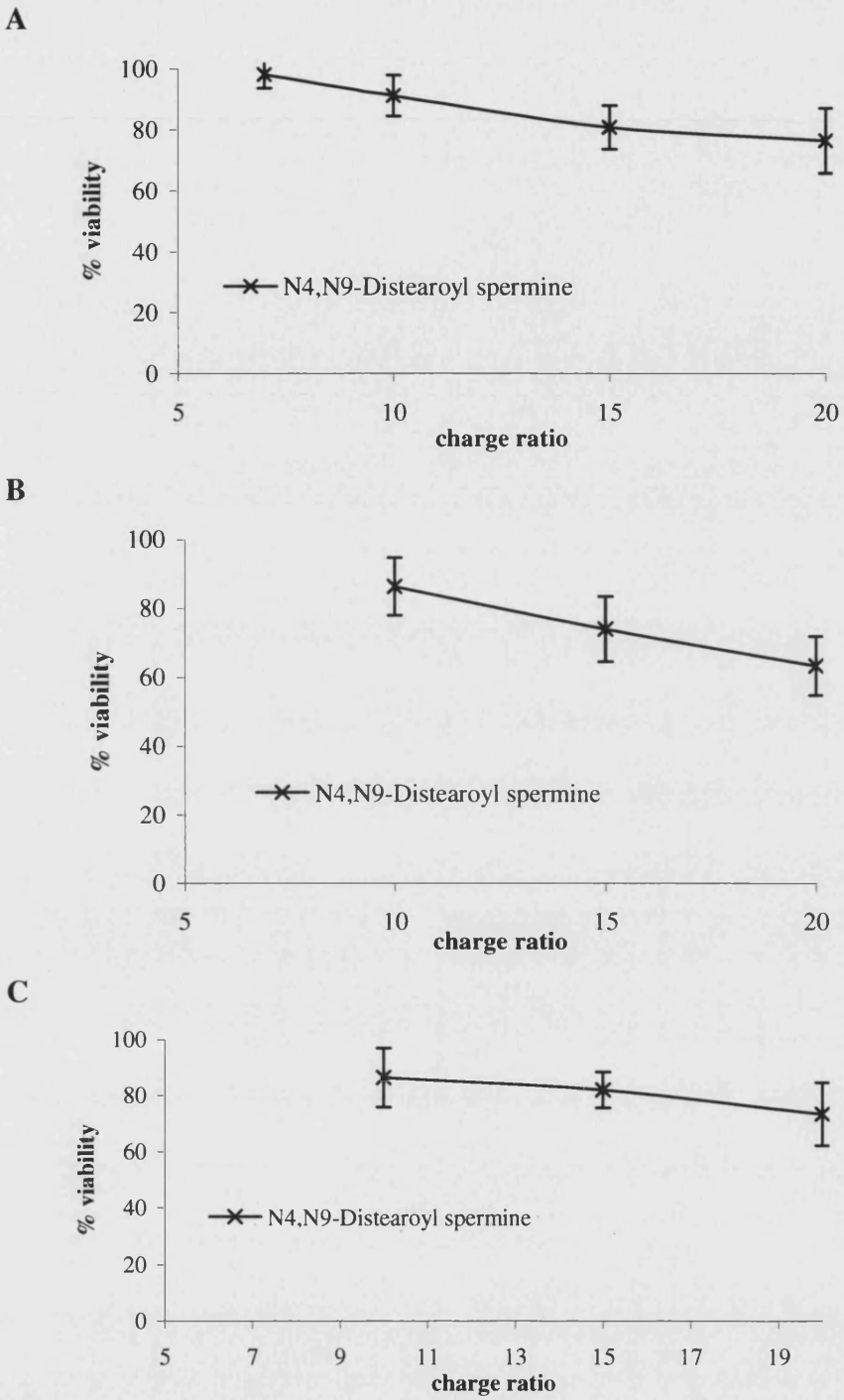


Figure 24. Viability of primary skin cell lines **A** FCP4, **B** FCP5 and **C** FCP7 cells after application of N^4,N^9-distearoyl spermine complexed with pEGFP at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

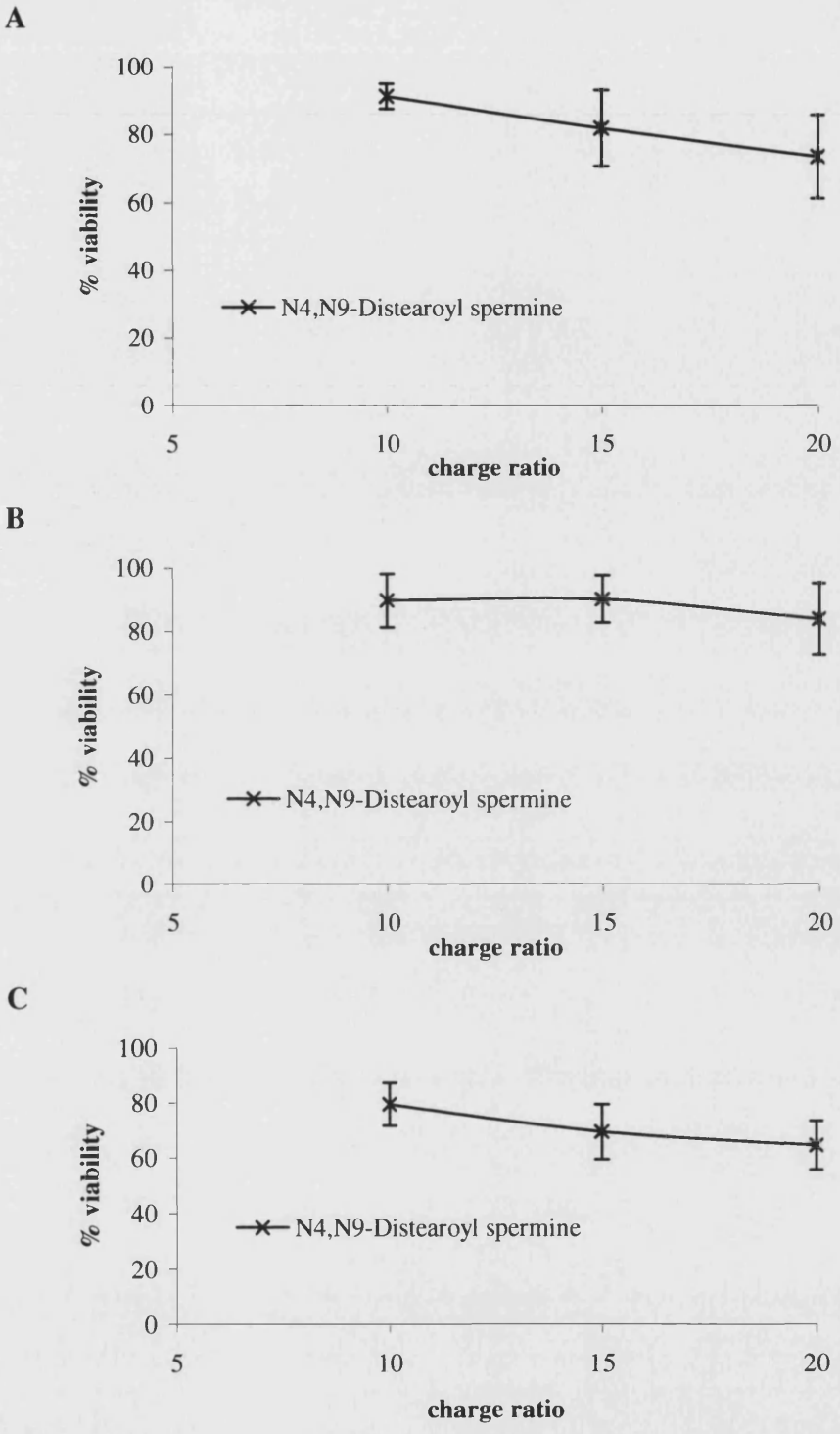


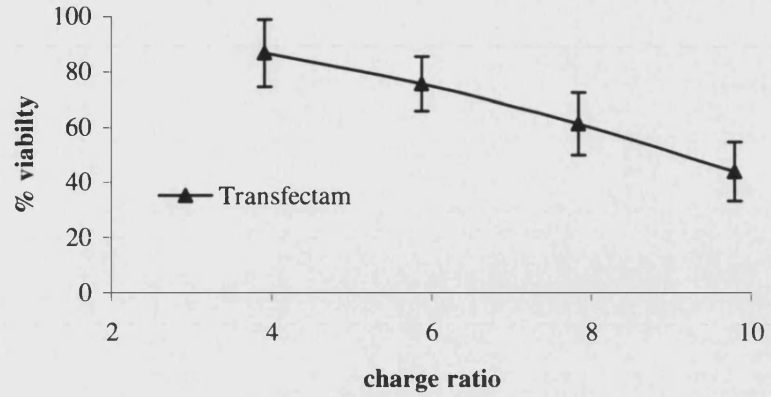
Figure 25. Viability of primary skin cell lines **A** FCP8 and **B** FEK4 and the cancer cell line **C** HtTA cells after application of N^4,N^9-distearoyl spermine complexed with pEGFP at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

Fig. 26 shows cell viability of FCP4, FCP5 and FCP7 primary cells after application of Transfectam® lipoplex at different charge ratios. The results show that 44 %, 39 %, 31 % cell viability at charge ratio 9.8 in the case of FCP4, FCP5 and FCP7, respectively Fig. 26. In the case of FCP8 and FEK4, Transfectam® lipoplex shows 49 % and 31 % cell viability at charge ratio 9.8, as shown in Fig. 27. In the case of HtTA cell line, as in the case of the primary cell lines, cell viability decreases with increase in the charge ratio and the lipoplex show 43 % at charge 9.8 Fig. 27.

In the case of N^4,N^9-dilinoleoyl spermine lipoplexes, viability of FCP4, FCP5 and FCP7 primary cells at different charge ratios show a decrease with increase in the charge ratio (Fig. 28). The results show FCP4 cell viability of 29 % at charge ratio 7.5 and 44 % and 54 % cell viability at charge ratio 6.5 in the case of FCP5 and FCP7 respectively (Fig. 28). The increased toxicity levels of the lipoplex could be attributed to the high efficiency of the lipopolyamine in transfecting the primary and cancer cell lines investigated.

In the case of the primary cell lines FCP8 and FEK4, cell viability results in Fig. 29 show 41 % and 43 % at charge ratio 7.5, respectively. In the case of HtTA cell line, N^4,N^9-dilinoleoyl spermine lipoplexes show cell viability of 53 % at charge ratio 6.5.

A



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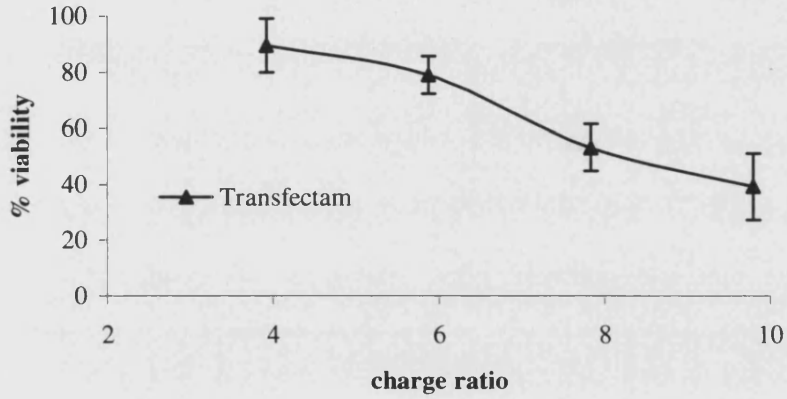

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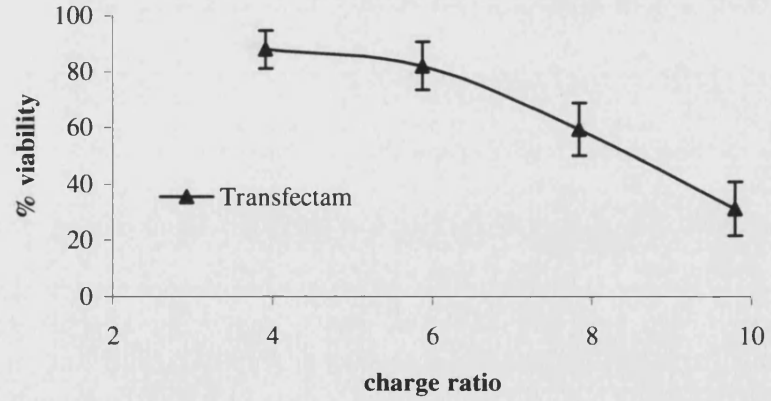
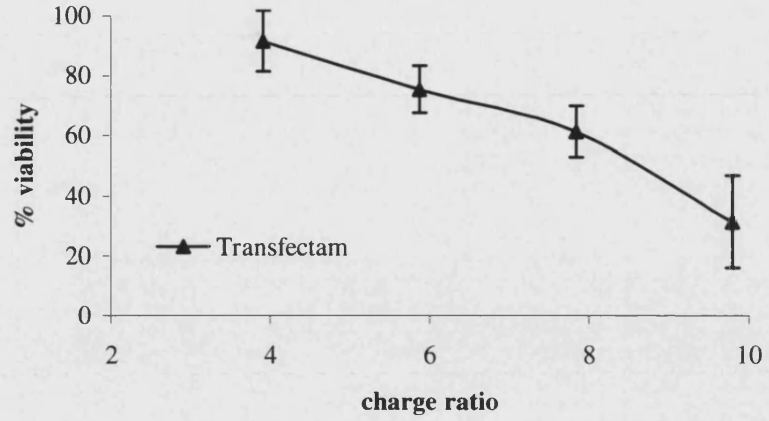


Figure 26. Viability of primary skin cell lines **A** FCP4, **B** FCP5 and **C** FCP7 cells after application of Transfectam® complexed with pEGFP at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.



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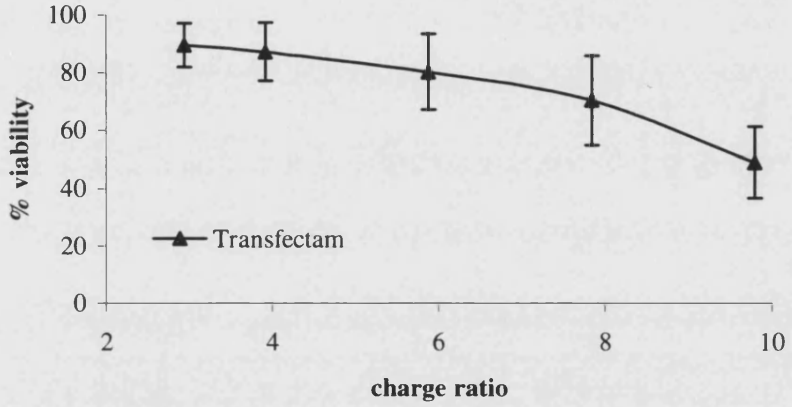

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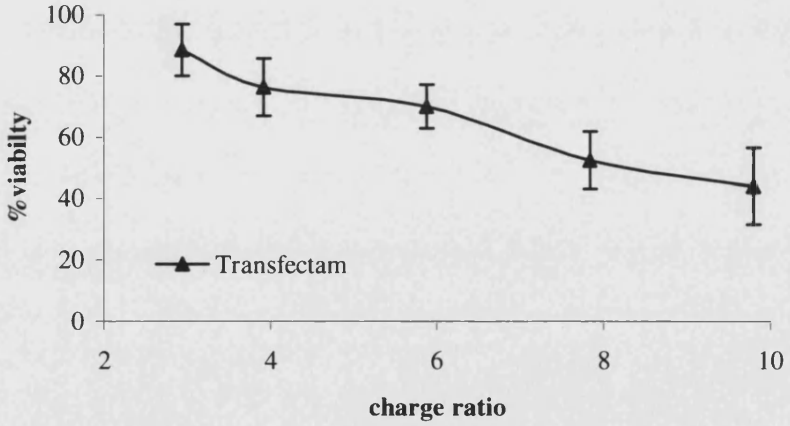
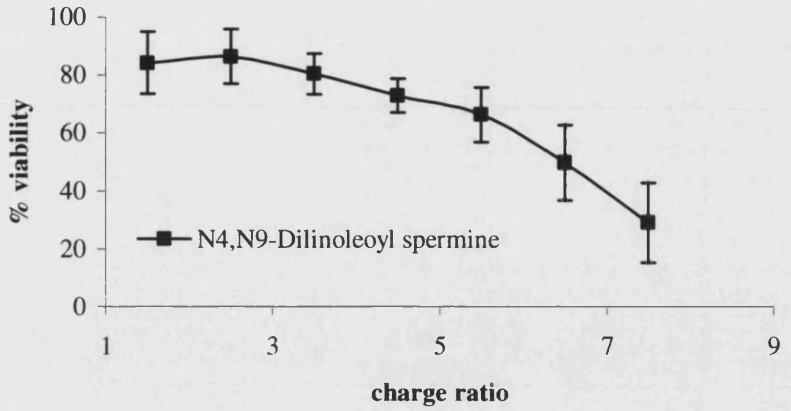


Figure 27. Viability of primary skin cell lines **A** FCP8 and **B** FEK4 and the cancer cell line **C** HtTA cells after application of Transfectam® complexed with pEGFP at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.



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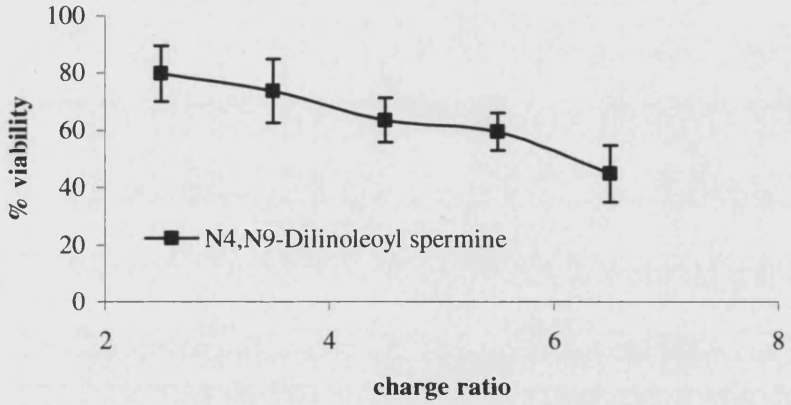

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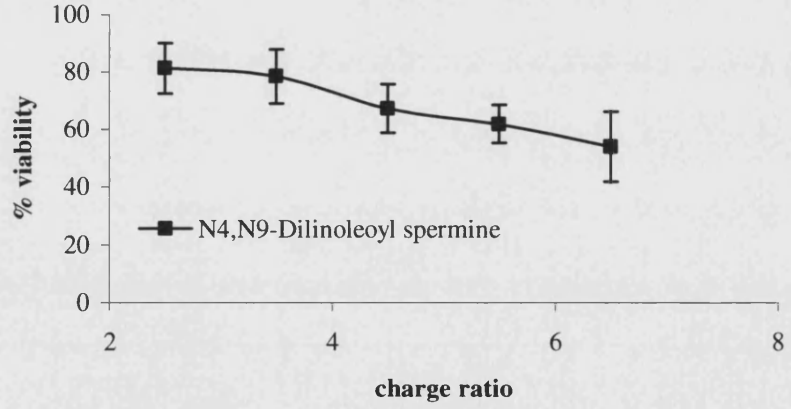


Figure 28. Viability of primary skin cell lines **A** FCP4, **B** FCP5 and **C** FCP7 cells after application of N^4,N^9-dilinoleoyl spermine complexed with pEGFP at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

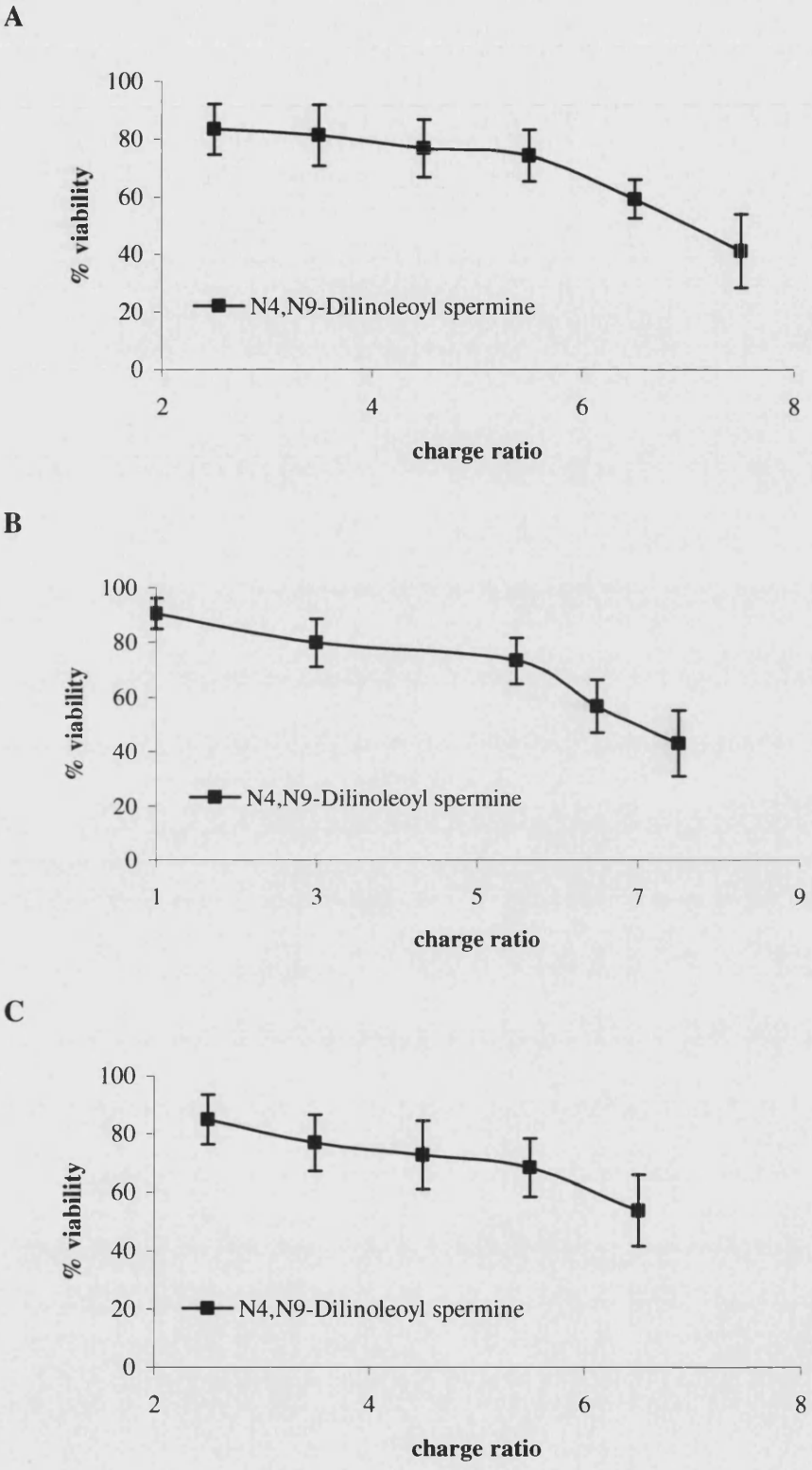


Figure 29. Viability of primary skin cell lines **A** FCP8 and **B** FEK4 and the cancer cell line **C** HtTA cells after application of N^4,N^9-dilinoleoyl spermine complexed with pEGFP at different charge ratios. The data show 3 different experiments (3 replicates each) and the error bars are the standard deviation.

Fig. 30 shows the transfection efficiency results in relation to cell viability levels at different charge ratios N^4,N^9-distearoyl spermine for both the primary cell lines and the cancer cell line HtTA in order to determine the optimum charge ratio (concentration) of transfection. The optimum charge ratio is 15 (33.4 μg/ml).

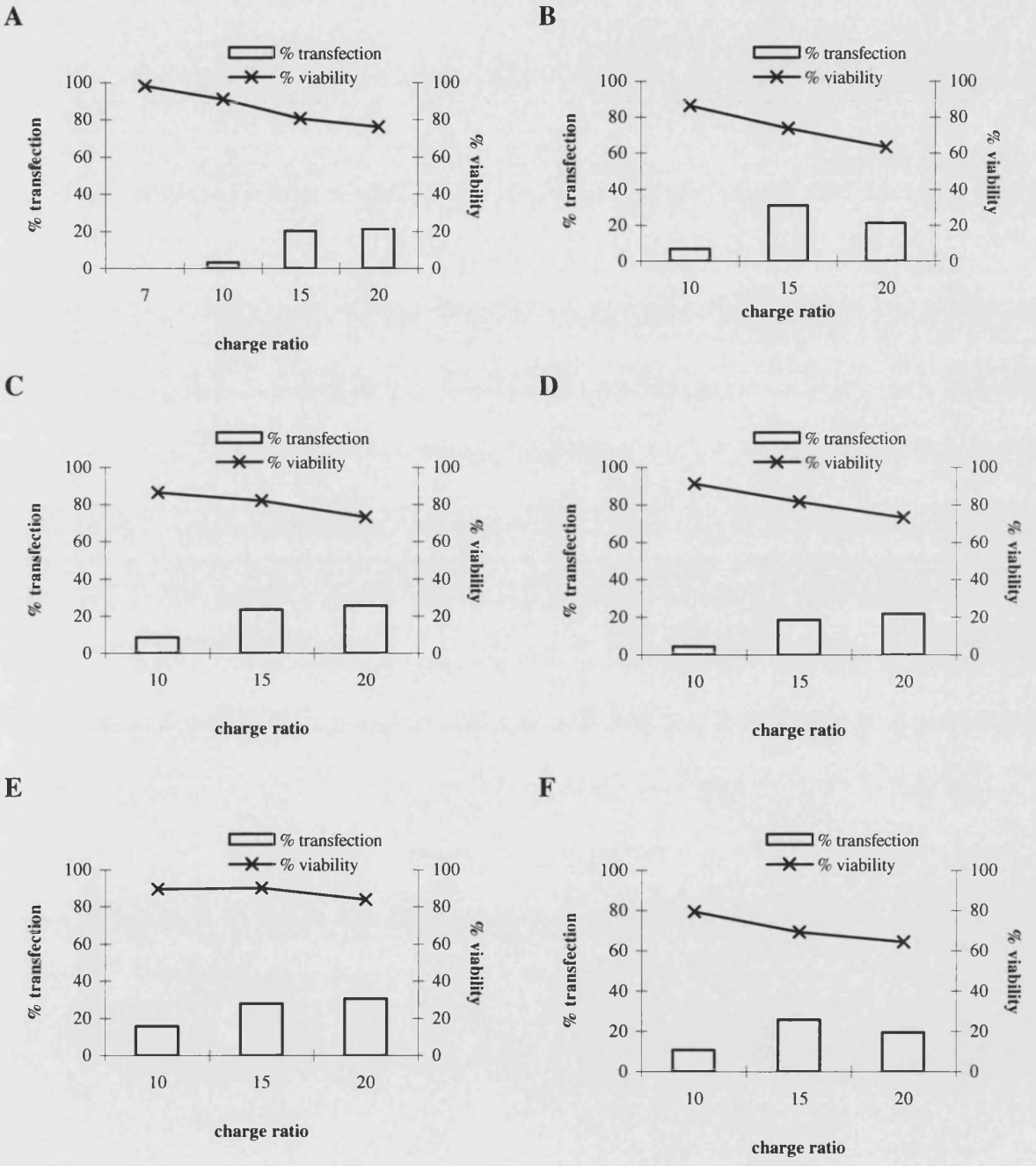


Figure 30. Lipofection and cytotoxicity studies of the primary cells **A** FCP4, **B** FCP5, **C** FCP7, **D** FCP8 and **E** FEK4 and **F** the carcinoma cell line HtTA transfected with pEGFP complexed with N^4,N^9-distearoyl spermine at different N/P charge ratios.

In the case of Transfectam® lipoplexes, the transfection efficiency results in relation to cell viability levels at different charge ratios show that the optimum charge ratio of transfection is 5.9 (15.0 µg/ml) as shown in Fig. 31.

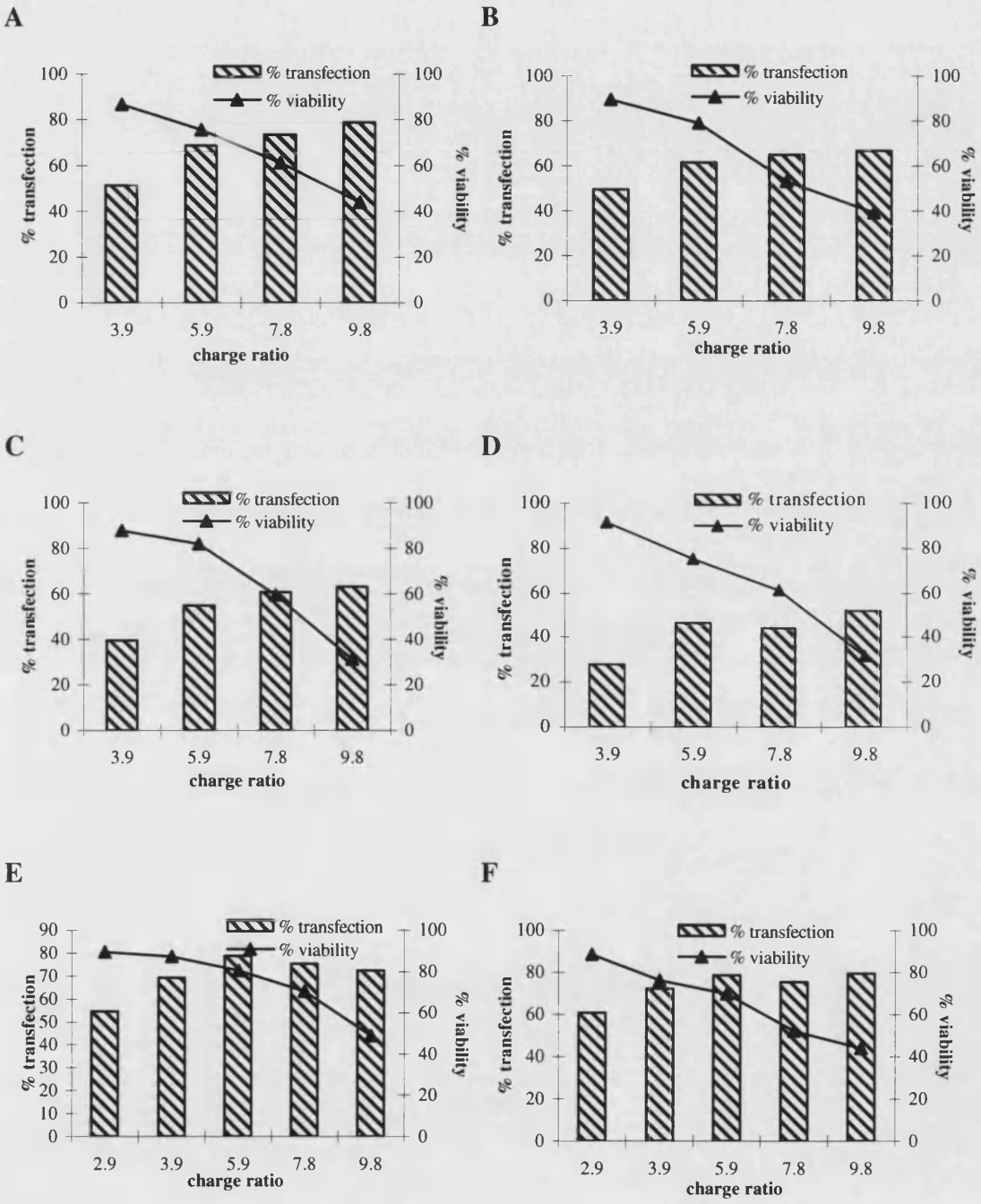


Figure 31. Lipofection and cytotoxicity studies of the primary cells **A** FCP4, **B** FCP5, **C** FCP7, **D** FCP8 and **E** FEK4 and **F** the carcinoma cell line HtTA, transfected with pEGFP complexed with Transfectam® at different N/P charge ratios.

The optimum N/P charge ratios (and corresponding concentrations) for transfection for N^4,N^9-dilinoleoyl spermine lipoplexes in primary and HtTA cell lines were experimentally determined to be N/P = 5.5 (12.1 μg/ml,) as shown in Fig. 32.

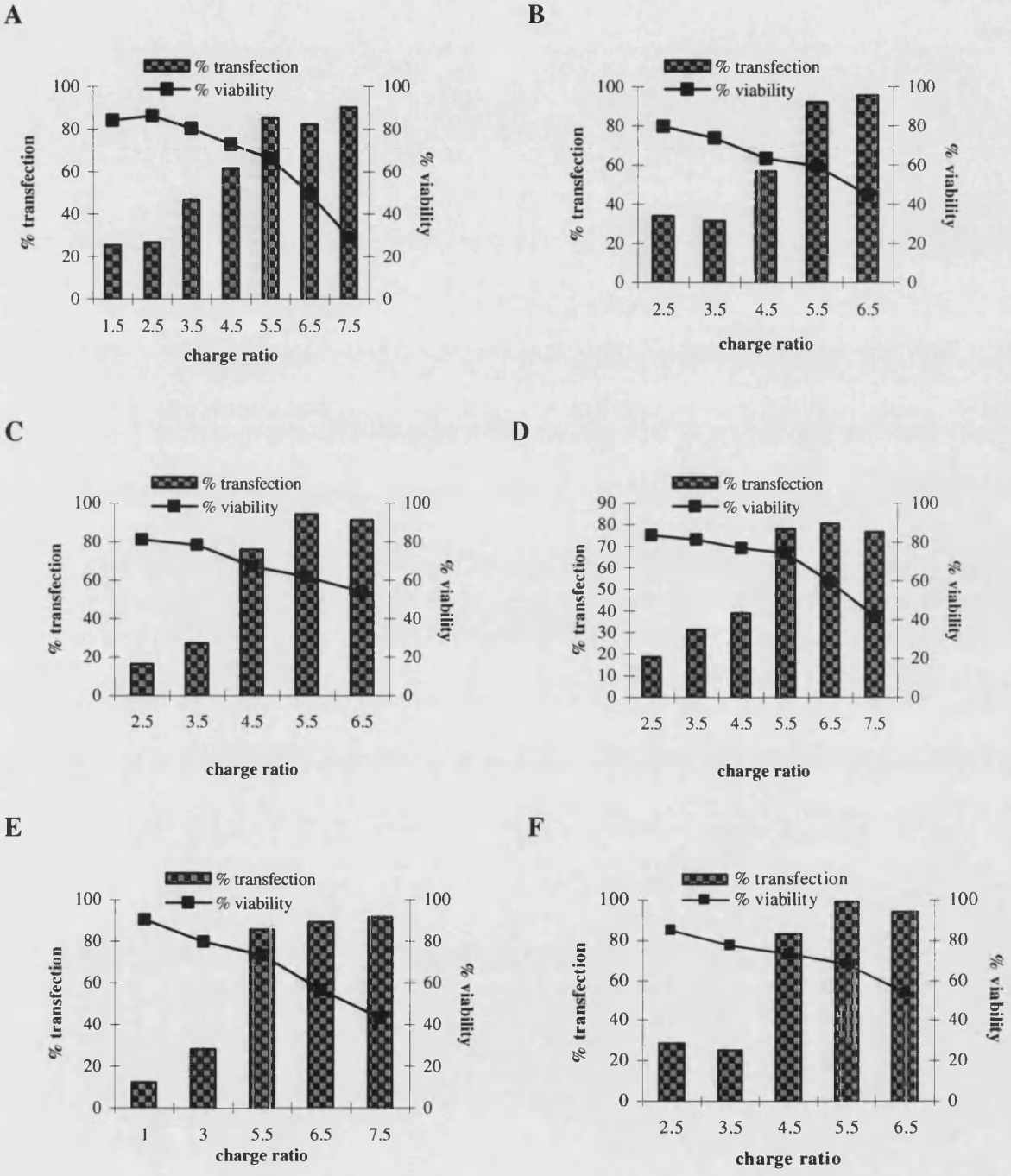


Figure 32. Lipofection and cytotoxicity studies of the primary cells **A** FCP4, **B** FCP5, **C** FCP7, **D** FCP8 and **E** FEK4 and **F** the carcinoma cell line HtTA, transfected with pEGFP complexed with N^4,N^9-dilinoleoyl spermine at different N/P charge ratios.

The transduction of pEGFP into a series of primary skin cell lines (FEK4, FCP4, FCP5, FCP7, and FCP8) and a cancer cell line (HeLa derived HtTA) was investigated using our novel lipopolyamines. The transfection results at their optimum charge ratio of transfection indicate the improved transfection ability of N^4,N^9-dilinoleoyl spermine, greater than 85 % in many of the studied primary cell lines (80 % for FCP8 cells) and 99 % in the case of the cancer cell line HtTA compared to the saturated N^4,N^9-distearoyl spermine (18-32 %), the mono-unsaturated N^4,N^9-dioleoyl spermine (62-75 %), and the commercially available Transfectam® (46-79 %) (Fig. 33).

As a negative control, naked (uncomplexed circular) pEGFP DNA typically gave 1-2 % transfection of these cell lines. Also, the shift in the EGFP positive cells compared to the untransfected cells in FACS analysis (Figs. 34 and 35), show the high efficiency of N^4,N^9-dilinoleoyl spermine of transfecting both primary (e.g. FEK4) and cancer (HtTA) cell lines.

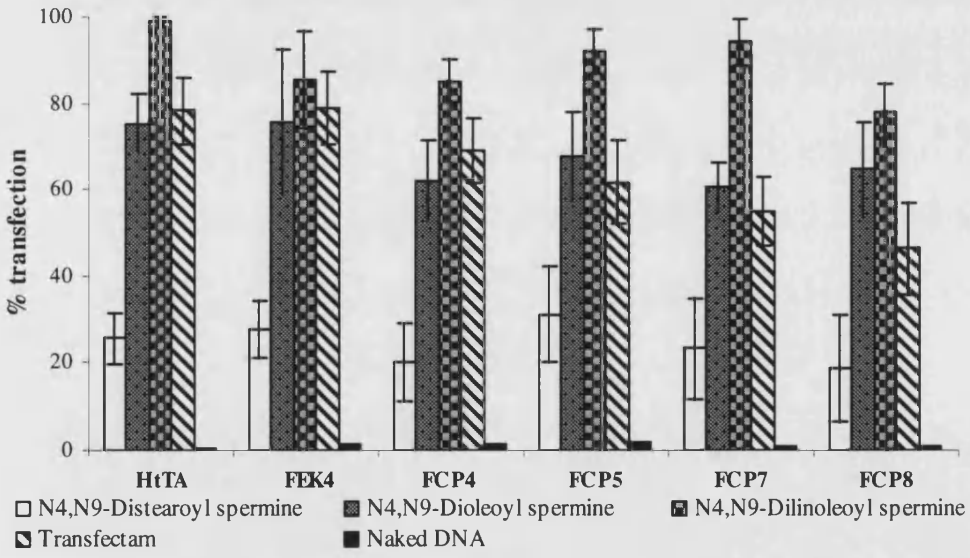


Figure 33. Lipofection of the primary skin cell lines (FEK4, FCP4, FCP5, FCP7, and FCP8) and the cancer cell line HtTA transfected with pEGFP complexed with N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine, N^4,N^9-dilinoleoyl spermine, and Transfectam® (at their respective N/P ratios for best transfection). The data show 3 different experiments (3 replicates each) and the error bars are the S.D.

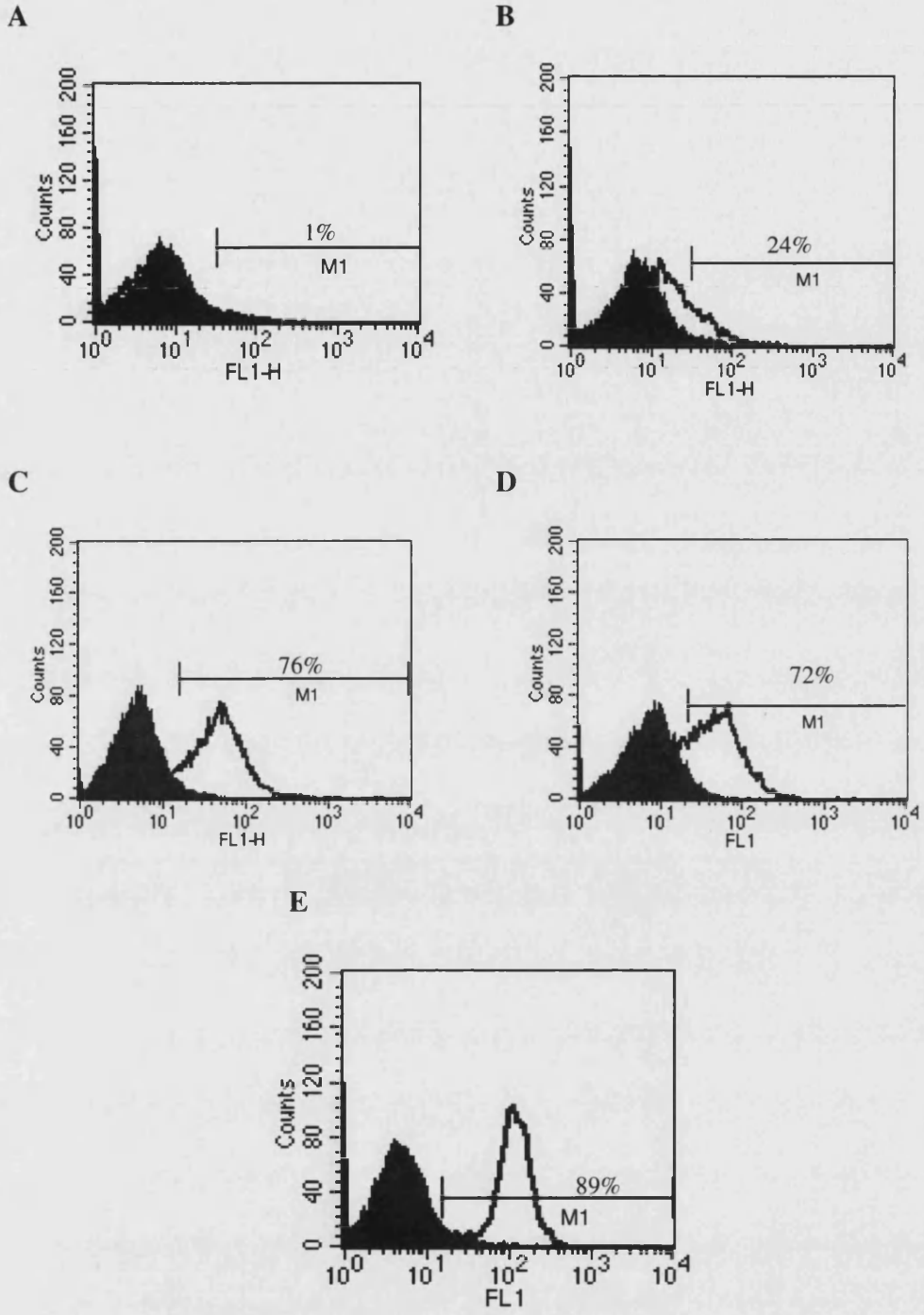


Figure 34. FACS analysis of FEK4 cells after 48 h transfection of pEGFP either **A** free or complexed with **B** N^4,N^9-distearoyl spermine, **C** N^4,N^9-dioleoyl spermine, **D** N^4,N^9-dilinoleoyl spermine or **E** Transfectam®: ■ untransduced cells, □ EGFP positive cells, M_1 is the fluorescence intensity range.

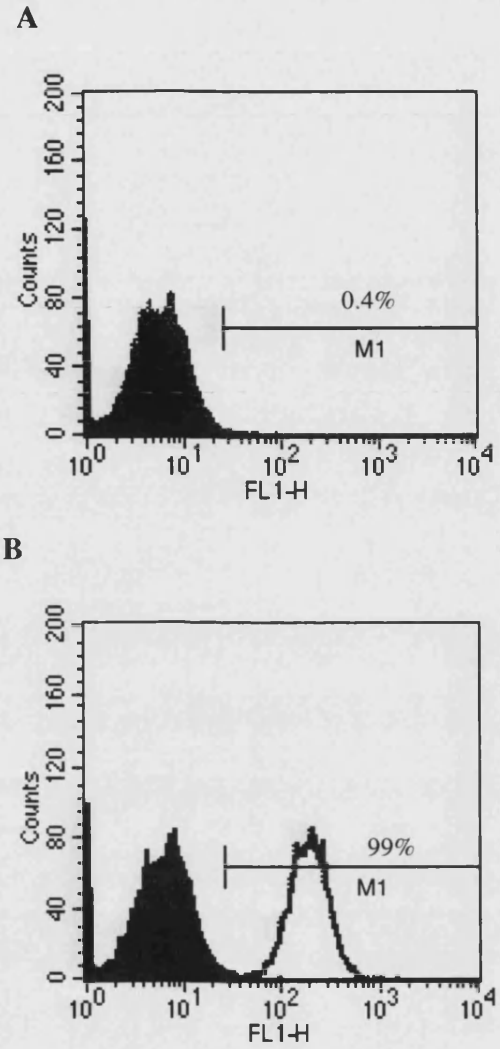


Figure 35. FACS analysis of HtTA cells after 48 h transfection of pEGFP either **A** free or complexed with **B** N^4,N^9-dilinoleoyl spermine (at the optimum charge ratio of transfection, 5.5): ■ untransduced cells, □ EGFP positive cells, M_1 is the fluorescence intensity range.

Cell viability (MTT assay (240)) results indicate that there is no significant difference in lipoplex toxicity, at the respective N/P ratios for best transfection, in the case of HtTA and FCP8 primary skin cells for all four studied lipopolyamines, N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine, N^4,N^9-dilinoleoyl spermine and Transfectam® with percentage cell viability about 70 % and 75 % in the case of the cancer cell line HtTA and the primary skin cells FCP8, respectively (Fig. 36). Only small differences were observed across the other four primary skin cell lines (FEK4, FCP4, FCP5 and FCP7) often with percentage cell viability about 80 %.

The lipopolyamine N^4,N^9-dilinoleoyl spermine that show the best transfection results of the investigated lipopolyamines, typically shows 70 % cell viability in most of the studied cell lines, and such a value has recently been reported to be acceptable for a safe DNA delivery vector (258). The cytotoxicity results of the lipoplexes were compared with the viability of both primary and cancer cell lines transfected with uncomplexed (naked) pEGFP (Fig. 36).

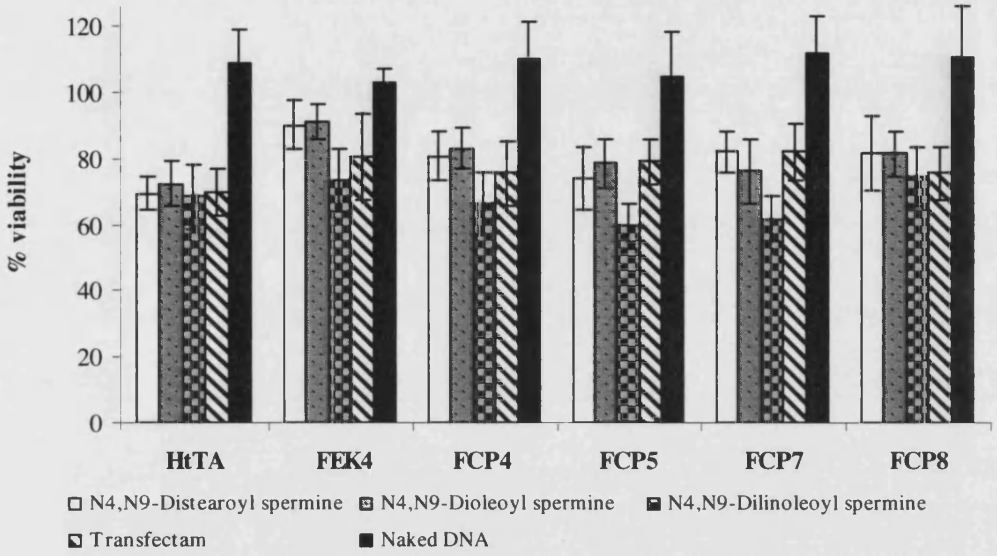


Figure 36. Cytotoxicity effect of pEGFP (2 μg/ml) free (naked DNA) or complexed with N^4,N^9-distearoyl spermine (33.4 μg/ml), N^4,N^9-dioleoyl spermine (5.5 μg/ml), N^4,N^9-dilinoleoyl spermine (12.1 μg/ml) and Transfectam® (15.0 μg/ml) in the tested cells.

Characterization of the lipoplex formulations

Lipoplex particle size and zeta potential measurement

Condensation of DNA into nanoparticles is a way to decrease the size of the delivered gene in order to facilitate cellular membrane entry by endocytosis and subsequent trafficking to the nucleus. Zeta potential characterizes the stability of the formulation. Romoren et al. (141) investigated the long-term stability of chitosan-based polyplex solutions (25 mM sodium acetate buffer, pH 5.5) at different

temperatures (4 °C, 25 °C, 45 °C) over a period of up to 1 year. They showed the relation between the change in the physicochemical characteristics of the polyplexes (particle size and zeta potential) and the change in the transfection efficiency.

Lipoplex particle size measurement

The average particle size for the lipoplexes formed (at their optimum charge ratio of transfection) between plasmid DNA and our novel synthetic lipopolyamines, after mixing with vortex mixer was determined using Malvern Zetasizer (Nano S, Malvern Instruments). All measurements were carried out on lipoplexes having a 5 μg/ml plasmid DNA in HEPES buffer at pH 7.4 at room temperature.

The characterization of particle size by dynamic light scattering revealed that the average particle size of both N^4,N^9-dilinoleoyl spermine and Transfectam® were considerably smaller (71 and 62 nm, respectively) than that of N^4,N^9-distearoyl spermine and N^4,N^9-dioleoyl spermine that show increased average particle size of 217 and 366 nm, respectively, as indicated in Table 2. Polydispersity of size ranged from 0.15 in the case of N^4,N^9-dilinoleoyl spermine and Transfectam, to 0.4 in the case of N^4,N^9-distearoyl spermine and N^4,N^9-dioleoyl spermine (Table 2). All the particle size measurements were carried out on the lipoplexes at their optimum charge ratio of transfection.

The results of pEGFP (4.7 kbp) condensation and formation of 62 nm nanoparticles with Transfectam® were in agreement with the results found by Dunlap et al. in their study of the commercial lipospermine condensation of plasmid DNA (5–7 kbp) that induced condensates of 50–70 nm in diameter (259).

It was noticed from the titration of the cationic lipids N^4,N^9-dioleoyl spermine and N^4,N^9-dilinoleoyl spermine with pEGFP that the complexes formed were small (about 100 nm) at low charge ratio (+/-, N/P) and grew to more than 1μm with increasing the charge ratio (about 2) for both cationic lipids , followed by a decrease in the particle size with a further increase in the charge ratio.

These results were in agreement with the results of using cationic polymers by Pouton et al. (260), the lipopolyamines RPR128522 and RPR120535 (2-(3-[4-(3-amino-propylamino)-butylamino]-propylamino)-*N*-dioctadecylcarbamoylmethyl-acetamide) by Byk et al. (50), DOTMA (2,3-dioleyloxypropyl-1-trimethylammonium chloride)/DOPE (dioleoyl phosphatidylethanolamine) liposomes by Sakurai et al. (261) that show increase in the particle size (up to a micron size level) of the condensed DNA with increase in the ratio of the condensing agent to DNA, followed by decrease in the particle size with further increase in the ratio.

The appearance of large particles reflected an aggregated form of the lipoplexes formed. Choosakoonkriang et al. (262) reported that the size of the cationic lipids/DNA complexes, DOTAP and DDAB (dioctadecyldimethylammonium bromide), was constant (150 nm) until ratio 0.5 followed by a gradual increase in the size until ratio 1.5 then a large increase (>1 μm) in particle size above 1.5 (lipid/DNA), the same was noticed after incorporation of the helper lipids cholesterol and DOPE.

Table 2. Particle size and polydispersity of DNA lipoplexes of N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine, N^4,N^9-dilinoleoyl spermine and Transfectam® at their optimum charge ratio for transfection.

Lipospermine	Charge ratio (N/P)	Lipoplex diameter (nm)	Poly-dispersity
N^4,N^9-Distearoyl spermine	15.0	217 (21)	0.46
N^4,N^9-Dioleoyl spermine	2.5	366 (73)	0.32
N^4,N^9-Dilinoleoyl spermine	5.5	71 (2.5)	0.19
Transfectam®	5.9	62 (3.2)	0.22

Each lipoplex diameter value shows the mean ± S.D.

Zeta potential measurement

The average ζ-potential measurement for the lipoplexes formed (at their optimum charge ratio of transfection), after mixing with vortex mixer, was determined using a Malvern Zetasizer (Nano ZS, Malvern Instruments). All measurements were carried out on lipoplexes having a 5 μg/ml plasmid DNA in HEPES buffer at pH 7.4 at room temperature.

The results revealed that the surface charge, as determined by ζ-potential measurements, was +43 and +32 mV for N^4,N^9-dilinoleoyl spermine (at N/P charge ratio of 5.5) and N^4,N^9-dioleoyl spermine (at N/P charge ratio of 2.5) lipoplexes, respectively. Transfectam® lipoplex shows a ζ-potential of +20 mV (at N/P charge ratio of 6) in Phosphate Buffered Saline (PBS) solution (Remy and Behr, personal communication). Also, from the titration of the cationic lipids N^4,N^9-dioleoyl spermine and N^4,N^9-dilinoleoyl spermine with pEGFP, these results show that the complexes show a negative ζ-potential at low charge ratio (+/-, N/P), -45 mV at charge ratio 1 in the case of N^4,N^9-dioleoyl spermine and -50 mV at charge ratio 0.9 in the case of N^4,N^9-dilinoleoyl spermine. Then, the ζ-potential increases with the increase in charge ratio up to charge ratio about 2 where the ζ-potential reaches the charge neutrality (zero). After that, the ζ-potential increases to positive values with increase in the charge ratio more than charge ratio 2, as shown in Fig. 37.

From these titration results, it is revealed from both particle size and ζ-potential measurements that the appearance of large particle sizes, more than 1 μm at charge ratio about 2, reflected an aggregated form of the lipoplexes formed that could be explained from ζ-potential results as a result of charge neutrality on the surface of the lipoplexes nanoparticles. The value of the ζ-potential was close to zero (Fig. 37) at the same charge ratio of 2 that shows the largest increase in the particle size of the lipoplex.

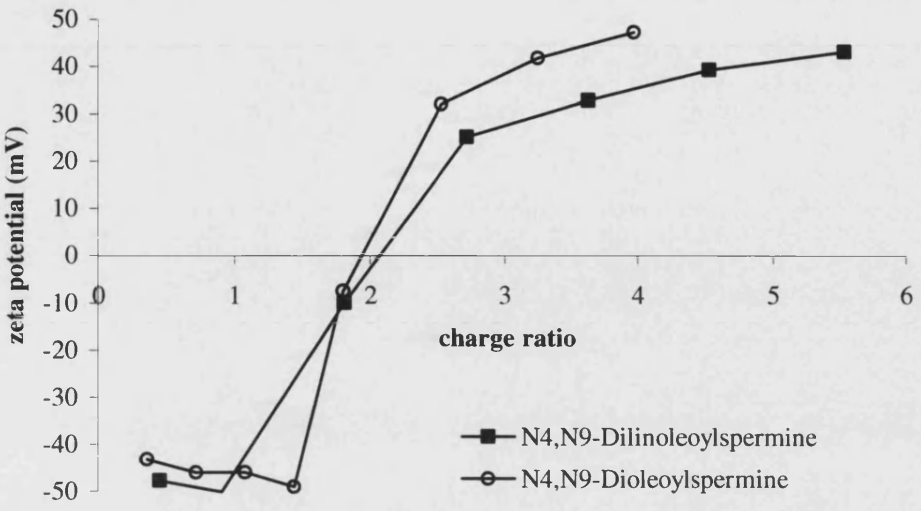


Figure 37. Zeta potential measurements of pEGFP with N^4,N^9-dioleoyl spermine and N^4,N^9-dilinoleoyl spermine measured at different (+/-) charge ratios.

Zeta potential is an important parameter helping to predict the stability of the formulation as well as the ability of the positively charged particles to interact with cell membranes (263, 264). Zeta potential depends on several factors including: pH, ionic charge, ion size, and concentration of ions in solution (265). The interaction of DNA with cationic lipids leads to the formation of net charge on the surface of the formed nanoparticles, either positive or negative according to the DNA- cationic lipid charge ratio, which attracts counter ions close to the surface of the formed nanoparticles (an electrical double layer). Ions close to the surface of the particle are bound relatively strongly to the surface until the hydrodynamic plane of shear (Fig. 38). After this plane, ion distribution is determined by a balance of electrostatic forces and random thermal motion. The potential in this region, therefore, decays as the distance from the surface increases until, at sufficient distance, it reaches the bulk solution value (zero). The potential at this imaginary surface, the hydrodynamic plane of shear, which separates the thin layer of liquid bound to the solid surface and showing elastic behaviour from the rest of the liquid is called the zeta (ζ) potential.

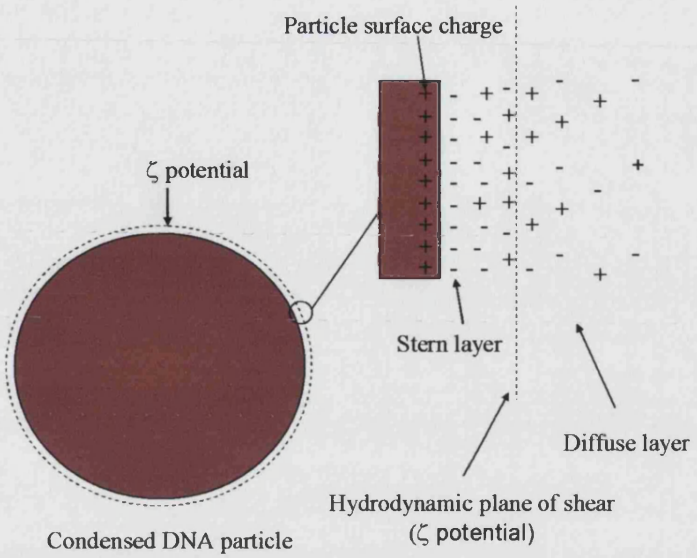


Figure 38. Diagram indicating the distribution of charges from the surface of the condensed DNA nanoparticle and the ζ potential.

The formed nanoparticles are considered to be stable when they have pronounced ζ-potential values, either positive or negative, but the tendency to aggregate is higher when the ζ-potential is close to zero. Charge neutrality ($\zeta = 0$) occurred at an N/P charge ratio of about 2 for both lipopolyamines N^4,N^9-dilinoleoyl spermine and N^4,N^9-dioleoyl spermine (comparable with our EthBr results, Fig. 3).

Martini and co-workers found neutralization N/P charge ratios of 6 and 2 with intact liposomes (265). Similarly, also working with liposomes, Middaugh and co-workers reported that neutralization charge ratios of cationic lipid-DNA complexes occurred at N/P charge ratios of 1.5, 2.5, and 3.5 (266, 267). These results are in agreement with the studies of Yi et al. (268) and Sakurai et al. (261) where they used DOTAP (*N*-[1-(2,3-dioleoyloxy) propyl]-*N,N,N*-trimethylammonium methylsulfate) as the cationic lipid, and also in agreement with the results from an artificial lipoprotein used by Alanazi et al. (269) and with the results of Tang and Szoka found with their cationic polymers (270).

Agarose gel electrophoresis

The complex formation of our three synthetic lipopolyamines and the commercially available lipospermine Transfectam® with polyanionic DNA (pEGFP) was examined by analysis of the electrophoretic mobility of the circular plasmid DNA-lipopolyamine complex within an agarose gel (1 %) stained with EthBr. The unbound free DNA in the agarose gel was visualized under UV using GeneGenius (Syngene).

All four spermine conjugates (including Transfectam®) were able to condense pEGFP DNA efficiently (as a result of the interactions between the lipopolyamine ammonium ions and the DNA phosphate charges) at their optimised respective charge ratios (N/P) of transfection (see: Table 2) leads to immobilization and reduction of EthBr intercalation with DNA. All the tested lipopolyamines were able to completely inhibit the migration of the circular plasmid DNA from lipoplexes in the agarose gel at their respective charge ratio of transfection as indicated in Fig. 39.

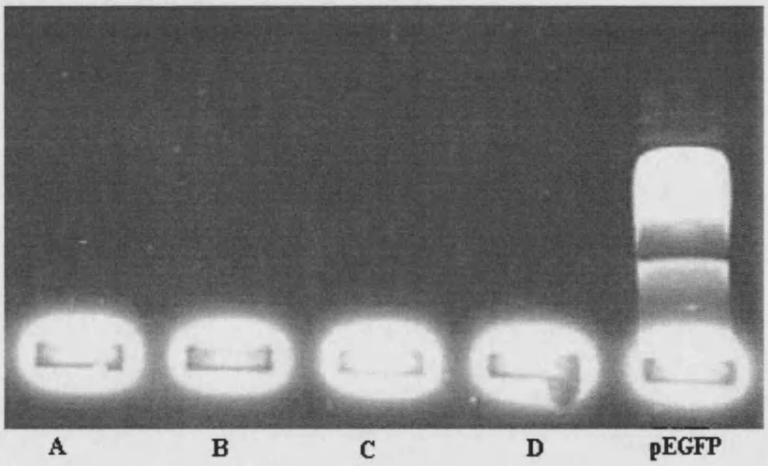


Figure 39. Gel retardation assay of pEGFP either free (pEGFP lane) or complexed with N^4,N^9-distearoyl spermine (lane A), N^4,N^9-dioleoyl spermine (lane B), N^4,N^9-dilinoleoyl spermine (lane C), and Transfectam® (lane D) at their respective N/P ratios for best transfection.

Also, Fig. 40 shows the interaction of pEGFP with N^4,N^9-dilinoleoyl spermine at different (N/P) charge ratios of 0.25, 0.5, 1.0, 1.5, 2, 3 and 5. The results revealed

the ability of N^4,N^9-dilinoleoyl spermine to completely immobilize pEGFP at higher charge ratios (charge ratio 3 and 5). While at lower charge ratios (≤ 2) a moderate inhibition of pEGFP in the agarose gel was observed (Fig. 40). These results were in agreement with the EthBr fluorescence intensity (DNA condensation) results (Fig. 3) that showed more than 90 % fluorescence quenching was achieved by pEGFP-N^4,N^9-dilinoleoyl spermine charge ratio of 2. This value (more than 90 % fluorescence quenching) achieved by N^4,N^9-dilinoleoyl spermine indicates the complete condensation of pEGFP (19, 22).

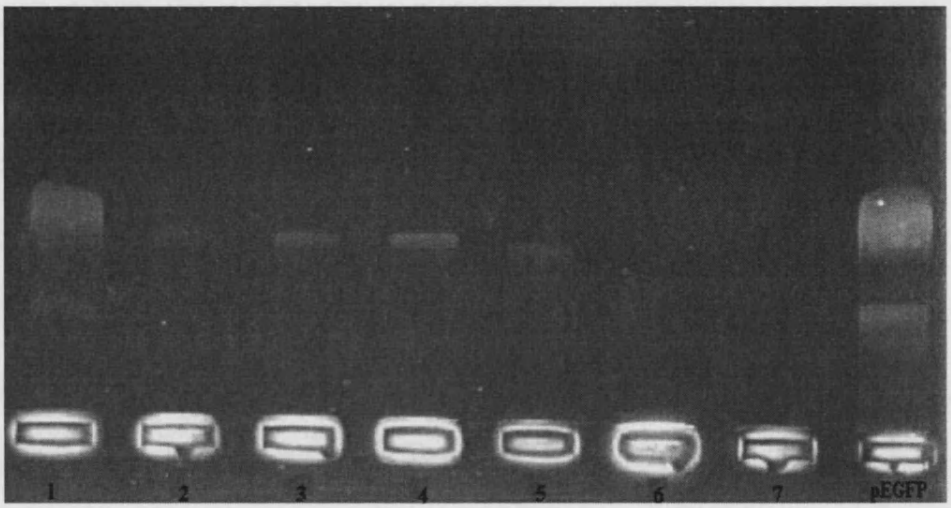


Figure 40. Gel retardation assay of pEGFP either free (pEGFP lane) or complexed with N^4,N^9-dilinoleoyl spermine at different lipopolyamine/ DNA ratios (N/P ratios), N/P 0.25 (lane 1), N/P 0.5 (lane 2), N/P 1.0 (lane 3), N/P 1.5 (lane 4), N/P 2.0 (lane 5), N/P 3.0 (lane 6) and N/P 5.0 (lane 7).

In this study, we have investigated the change in the degree of unsaturation of the di-C18 fatty chain formulation of lipospermine on DNA condensation and cellular delivery. The results from pEGFP condensation, investigated by EthBr fluorescence quenching assay, revealed that two of our synthetic lipopolyamines and the commercial formulation Transfectam® were able to condense DNA to less than 10 % EthBr fluorescence, where DNA is defined as condensed (19), however N^4,N^9-distearoyl spermine had not reduced (quenched) the EthBr fluorescence to 10 % by N/P charge ratio 3.5 (Fig. 3). Particle size of the final gene formulation is also an important factor in improving gene delivery (141, 271). Particle size results (Table 2)

showed larger particles with both N^4,N^9-dioleoyl spermine and N^4,N^9-distearoyl spermine formulations over those obtained with N^4,N^9-dilinoleoyl spermine and Transfectam®. These results are in concordance with the improvement in transfection achieved with N^4,N^9-dilinoleoyl spermine over our other two formulations.

On the other hand, although Transfectam® has the smallest particle size (62 nm), the saturation of the di-C18 fatty chains could possibly contribute to the lower transfection efficiency of this spermine conjugate in comparison to our formulation results with N^4,N^9-dilinoleoyl spermine. Gao and Huang (272) reported that the addition of poly-L-lysine (PLL) or protamine to DC-Chol liposomes reduces the size of the complex as well as its heterogeneity in all ratios of lipid/DNA that improve transfection efficiency. Noguchi et al. (63), also working with protamine, reported that smaller sized particles transfect cells efficiently. The incorporation of protamine as a nuclear localization signal to liposomes incorporating a cationic cholesterol derivative reduced the size of the formed particles from 0.4-1.8 μm, for the DNA-liposome complex, to 0.1-0.8 μm for the DNA-protamine-liposome complex.

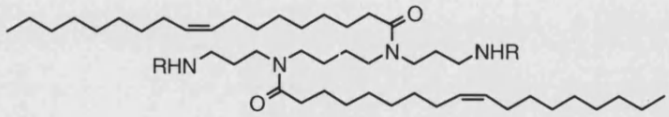
On the relationship between particle size and transfection efficiency, there are no definite limits to the nanoparticle size that are suitable for transfection (205). Nanoparticles have relatively higher intracellular uptake than microparticles (273). Also, on the nanoscale, smaller size polyplexes are more able to enter cells and thereby increase the efficiency of transfection (123). Similarly, in 2004, Hoekstra and co-workers showed with fluorescently labelled nanospheres (latex beads) that size itself can determine the pathway of entry (274). Wagner and co-workers reported larger particles (1000 nm) exceeding the transfection efficiency achieved with smaller particles (40 nm) using DNA/transferrin-polyethylenimine complexes at physiological salt concentration (275). With a different DNA condensation system, they also reported that small toroid structures of DNA/transferrin-PLL complexes (80-100 nm) showed high transfection efficiency as their diameters are in the range of the coated pits involved in the endocytosis process (276).

We have previously reported (145, 171, 241, 243-245) the importance of the substituents in the lipid moiety conjugated to the cationic polyamine in order to achieve improvements in DNA condensation efficiency for non-liposomal

formulations where the lipid moiety must be considered in shape (volume) and substituent pattern, as well as the polyamine moiety and its pK_a values. The design and synthesis of novel cationic lipids based upon the tetra-amine spermine, as non-liposomal formulations, where the lipid moiety is a long carbon chain was largely instigated by Behr and co-workers with their design and preparation of the highly efficient lipopolyamine dioctadecylamidoglycylspermine (DOGS, Transfectam®) (179, 185). A leading non-viral vector following from such structure-activity considerations is RPR120535 (182) where the di-C18 saturated alkyl chains are substituents pendant from a diamide at one end of the tetra-amine spermine, used as the cationic moiety to take advantage of DNA binding by polyamines (211).

Unsaturated chains have also been incorporated as the lipophilic moiety in lipopolyamine vectors. Kirby and co-workers (187) have used two covalently bound oleoyl chains in non-liposomal formulations for improved transfection efficiency by fusion with cellular membranes. They attached basic amino acids (Lys) to the primary groups (compounds type **A, 1** and **2**) or to the secondary amino groups of the lipospermine surfactants (compounds type **B, 3** and **4**) (Fig. 41). They found that Type **B** compounds with the same short peptides show higher transfection efficiency compared to type **A** compounds. Also, the use of unsaturated C18 oleoyl fatty chains shows the highest transfection efficiency and increasing the number of positive charges improves the ability of the compound as a non-viral gene delivery vector (187).

A **1** R =LysLysLysSer, **2** R = Lys



B **3** R =LysLysLysSer, **4** R = Lys

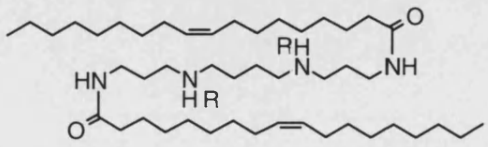
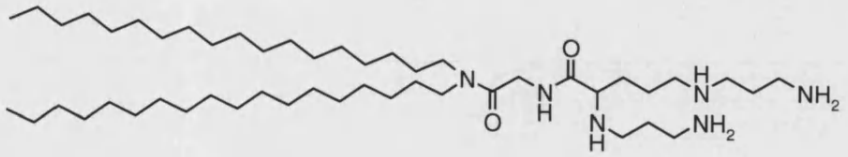
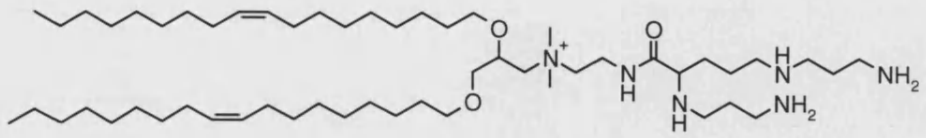


Figure 41. Lipospermine cationic surfactants.

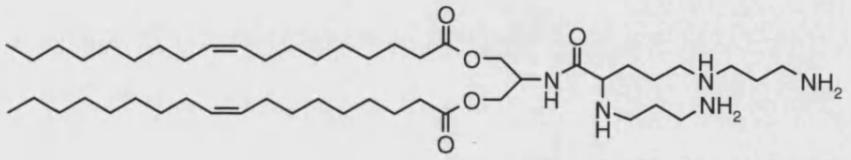
RPR120535



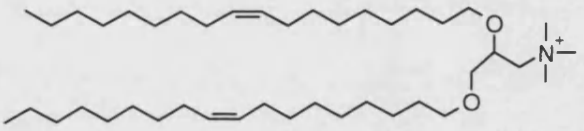
DOSPA



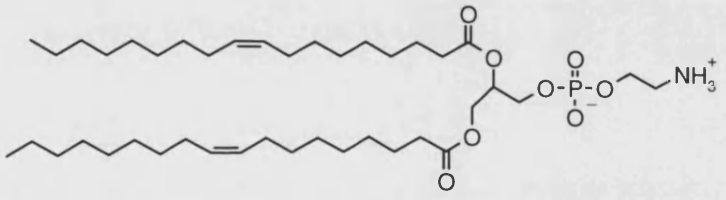
DOSPER



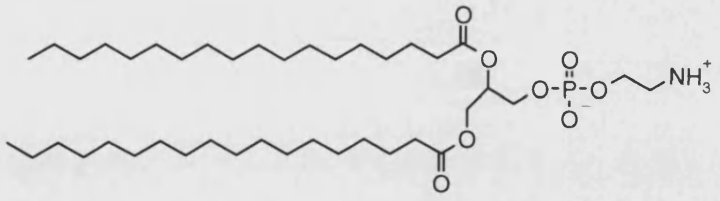
DOTMA



DOPE



PE



DOPC

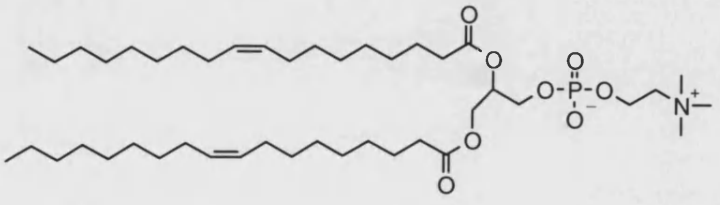


Figure 42. Spermine conjugates together with DOTMA (cationic) and neutral helper lipids, gene delivery formulation excipients.

In liposomal formulations, dioleoylphosphatidyl ethanolamine (DOPE) is often used as a helper lipid for liposomal formulation and for its fusogenic ability as it has the characteristics of non-bilayer forming activity leading to destabilisation of the lipid bilayer (209). Oleoyl and oleyl chains have also been bound as esters and ethers in liposomal formulations: 1,3-dioleoyloxy-2-(6-carboxyspermine) DOSPER (177), DOSPA-DOPE (3/1 w/w, Lipofectamine™), DOTMA-DOPE (1/1 w/w, Lipofectin®) (Fig. 42).

Non-liposomal cationic-lipid delivery vectors combine both the characteristics of cationic and of helper lipids. So a factor that is important for both facilitating cell entry and the efficient intracellular release of free DNA (or lipoplexes) into the cytoplasm is the influence of the cationic lipid chain. The lipid moiety in our cationic lipids interacts with the phospholipid bilayer of the cell membrane, and that, either in crossing the membrane bilayer and/or in helping to weaken the endosomal bilayer, thereby aids escape into the cytosol.

Cationic lipids in aqueous solutions exhibit different polymorphic phases. The two most important organizational forms are L_α (lamellar organization with fluid hydrocarbon chains) and H_{II} (two-dimensional hexagonal state) (Fig. 43) (277). Huang and co-workers have reported that the replacement of DOPE with a trimethylated structural analogue dioleoylphosphatidyl choline (DOPC) (Fig. 42), in cationic liposomal formulations, abolishes most of the transfection activity of the lipoplex, as DOPE exhibits a high tendency to form the inverted hexagonal (H_{II}) phase particularly at acidic pH (209).

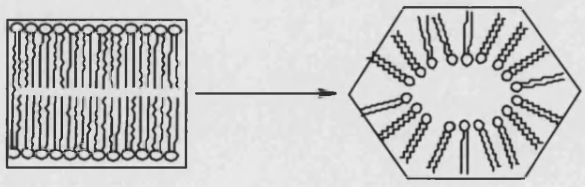
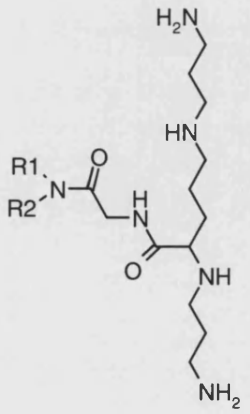


Figure 43. Typical diagram of the change in lipid phase structure from the lamellar L_α phase to the inverted hexagonal H_{II} phase.

Lipids that are cylindrical in solution shape prefer bilayer formations, while lipids with large head-groups and small diameter hydrocarbon chains (inverted cones) form micellar structures (277). Lipids with small head-groups and long hydrocarbon-chains e.g. (saturated) phosphatidyl ethanolamine (PE) and DOPE (Fig. 42), due to their solution cone shape, favour phase transition to an inverted hexagonal phase, as a result of steric factors (278). Also, increasing the degree of unsaturation of the acyl chain (especially in the *cis*-configuration) favours hexagonal phase formation ($L_\alpha \rightarrow H_{II}$ phase transition) and potentially improves the fusogenic characteristics of the lipid. The actual significance of maximising such fusogenic characteristics in NVGT remains to be proven.

Gaucheron, Santaella, and Vierling, in their detailed study on fluorinated lipospermines (Fig. 44), that are close analogues to Transfectam, speculated the improved transfection with the unsaturated over the saturated counterparts could be due to "their greater ability to promote membrane fusion with and destabilization of the endosomal membrane allowing optimal DNA release in the cytosol" and "related to the fluidification effect of the C=C bonds" (184). Vierling et al. have also reported (279) that unsaturated fluorinated lipids showed lower lamellar phase transition temperatures than the corresponding fluorinated saturated lipids. These results are in agreement with the higher transfection levels of the monounsaturated C18 (oleoyl group) over the saturated C18 (stearoyl) achieved by Felgner and his research group that was related to the possibility of the decrease in bilayer stiffness by introducing unsaturation into the alkyl chains (97).



R1 = $F(CF_2)_n(CH_2)_m$

$F(CF_2)_nCH{=}CH(CH_2)_m$

R2= $F(CF_2)_n(CH_2)_m$

$CH_3(CH_2)_x$

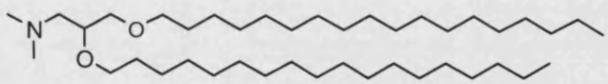
$F(CF_2)_nCH{=}CH(CH_2)_m$

R1 = R2 = $CH_3(CH_2)_{17}$ DOGS (Transfectam)

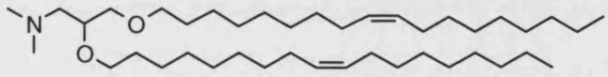
Figure 44. Fluorinated lipospermines (DOGS analogues).

In addition, the effect of degree of saturation of cationic lipid analogues was investigated to identify the fusogenic and transfection ability of these compounds in relation to their degree of unsaturation by Heyes and co-workers (176). The saturated cationic lipid (1,2-distearyloxy-*N*,*N*-dimethyl-3-aminopropane (DSDMA)) (Fig. 45) was more readily internalized to the cells, despite its almost no ability of gene silencing (siRNA delivery). While the unsaturated cationic lipids, the mono-unsaturated (1,2-dioleyloxy -*N*,*N*-dimethyl-3-aminopropane (DODMA)), the di-unsaturated (1,2-dilinoleyloxy-*N*,*N*-dimethyl-3-aminopropane (DLinDMA)) and the tri-unsaturated (1,2-dilinolenyloxy-*N*,*N*-dimethyl-3-aminopropane (DLenDMA)) (Fig. 45) show higher efficiency in gene silencing compared to the saturated DSDMA cationic lipid. Gene silencing increased with the increase of the degree of unsaturation, as a result of the increase in the fusogenic ability, lower transition temperature from L_α (lamellar organization) to H_{II} (two-dimensional hexagonal state), of the cationic lipid (176).

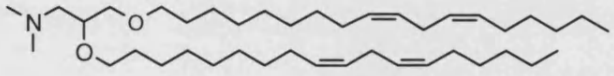
A



B



C



D

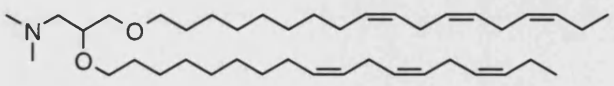


Figure 45. The cationic lipids, DSDMA **A**, DODMA **B**, DLinDMA **C** and DLenDMA **D**.

The fusogenic characteristics of our synthetic non-liposomal lipopolyamines were taken under consideration to account for the observed difference in gene delivery. The two factors head-group and chain-length are held constant among our investigated compounds. The degree of unsaturation of the C18 fatty chain may be a key factor that explains the increase in the transfection efficiency of the lipoplex. This may be a result of the improved fusogenic characteristics of N^4,N^9-dilinoleoyl spermine with its (di-*cis*-configuration) dienoic fatty acyl residue, more than the mono-unsaturated N^4,N^9-dioleoyl spermine and the saturated N^4,N^9-distearoyl spermine.

In conclusion, in this chapter we have designed, synthesized and characterised novel gene carriers. DNA condensation, particle size, transfection efficiency and cytotoxicity results revealed that N^4,N^9-dilinoleoyl spermine is a promising DNA delivery formulation with its increased degree of unsaturation in both of the lipospermine fatty chains. The dienoic C18 N^4,N^9-dilinoleoyl spermine achieves a high (85-90 %) transfection efficiency in primary cell lines and (99%) transfection efficiency in cancer cell line. The Transfection efficiency was in the order of the dienoic fatty acyl spermine conjugate N^4,N^9-dilinoleoyl spermine > the alkenoic N^4,N^9-dioleoyl spermine > the saturated N^4,N^9-distearoyl spermine. The increase in the degree of unsaturation (number of double bonds) in the fatty chains, due to the fluidification effect of the C=C bonds, improves the ability of the lipospermine to promote membrane fusion and destabilization of the endosomal membrane that ultimately increase the transfection efficiency of the cationic lipid.

Conclusions

From this thesis it can be concluded that new medicines can potentially be used to formulate and efficiently deliver DNA as the drug. In formulation terms, DNA is a medicinal substance that is difficult to formulate. This is because of the nature of both its size that is usually in Mega-Daltons and the many thousands of negative charges on the phosphate backbone of the polynucleotide. The use of an efficient carrier (delivery vehicle) responsible for the complex process of successful DNA delivery to the nucleus is a determinant factor for the successful application of gene therapy. DNA is condensed naturally by basic proteins, histones and by positively charged polyamines spermine and spermidine. Therefore, polycationic compounds (polyamines) were utilized in the design of our gene carriers.

Delivery vehicles for DNA formulation are either viral or non-viral vectors relying on (poly)cationic compounds. Non-viral formulation has to overcome cellular barriers to achieve a successful delivery of the prodrug DNA. These barriers include: DNA condensation, cell targeting, cell membrane entry, endosomal escape, nuclear entry, decomplexation, and transcription and translation. To overcome these barriers, several investigations were carried out to improve the delivery of DNA through several approaches including: the use of novel carriers, the delivery and internalisation to specific cells, incorporating moieties to improve the endosomal escape (e.g. proton sponge effect) or fusogenic lipids.

In these studies, the condensation of DNA and formation of nanoparticles was monitored using ethidium bromide (EthBr) fluorescence quenching, light scattering and gel electrophoresis assays. In addition, EthBr was used to determine the binding affinity of the condensing agents for DNA. The binding of cationic molecules with DNA is not entirely responsible for the release of EthBr, but alteration of the molecular flexibility of DNA through cationic compaction facilitated the release of bound EthBr. The number and/or the distribution of the positive charges on the molecule could have a significant effect on the affinity of the vector for DNA that leads to the efficient displacement of EthBr. A simple light scattering assay is useful for the detection of (nano)particle formation.

Transfection efficiency and cell viability (toxicity) experiments were carried out using pEGFP as a reporter gene, encoding for enhanced green fluorescent protein, either complexed with DNA condensing agents, or free (naked) DNA as a negative control. pEGFP was transformed and propagated in *E. coli* JM 109 bacterial strain. DNA yields and purity were determined spectroscopically (OD_{260}/OD_{280} = 1.80–1.90) and by agarose gel (1 %) analysis. Six primary cell lines were used in the transfection and cytotoxicity experiments, FEK4, FCP4, FCP5, FCP7 and FCP8 cells are human primary fibroblasts derived from newborn foreskin explants used as models for human primary cells. HeLa derivative and transformed cell line (HtTA) was used as a model for an immortal cancer cell line. The cytotoxicity (cell viability) of these compounds was studied in both primary skin and immortalised cancer cell lines using an MTT assay. The formed lipoplexes (nanoparticles) were characterized by both average particle size and ζ-potential measurements.

N^4,N^9-Dioleoyl spermine was synthesized from the naturally occurring polyamine spermine by primary amine protection using ethyl trifluoroacetate. DNA condensation and binding affinity results, using EthBr fluorescence quenching assay for N^4,N^9-dioleoyl spermine compared with model polyamines (spermine, PEI average molecular weight 2,000 Da (PEI 2k) and 60,000 Da (PEI 60k) and PLL average molecular weight 9,600 Da (PLL 9.6k) and 27,000 Da (PLL 27k)), revealed that addition of lipophilic moieties to the polyamine spermine improves its ability to condense DNA. No significant difference was detected in the condensation of linear (readily available calf thymus and herring testes) and circular plasmid (pEGFP and pβ-gal, which includes the gene *LacZ* encoding for β-galactosidase) DNA using EthBr fluorescence quenching assay. However, the type of formulation of the cationic lipid (liposomal or non-liposomal) could affect the DNA condensation efficiency. The results showed that N^4,N^9-dioleoyl spermine is able to condense DNA at a lower charge ratio than PLL 9.6K and spermine and produces a 50 % fluorescence decrease at charge ratio 0.52. N^4,N^9-Dioleoyl spermine showed improved DNA condensation ability in comparison with the commercially available, cationic lipid formulations Lipofectin® (DOTMA/DOPE 1/1 w/w) and Lipofectamine™ (DOSPA/DOPE 3/1 w/w).

The transfection of pEGFP (analysed by FACS) showed a higher transfection efficiency of N^4,N^9-dioleoyl spermine (at N/P charge ratio of 2.5) than that obtained using commercially available liposomal Lipofectin® formulation in FEK4 primary skin fibroblast and HtTA cell lines. On the other hand, there is no significant difference in the transfection activity between non-liposomal N^4,N^9-dioleoyl spermine and the commercially available standard liposomal formulation Lipofectamine™.

Cell viability results for N^4,N^9-dioleoyl spermine revealed improved FEK4 viability more than the cancer cells HtTA. Also, N^4,N^9-dioleoyl spermine showed a significant improvement in primary FEK4 cell viability over the liposomal formulations, but no significant difference in HtTA cells. N^4,N^9-Dioleoyl spermine shows promising transfection results in tissue cultured cancer and skin fibroblast primary cell lines. Therefore, we investigated the effects on DNA formulation with variation in the degree of unsaturation in the two C18 fatty chains of the lipospermine, preparing the novel saturated N^4,N^9-distearoyl spermine, alkenoic N^4,N^9-dioleoyl spermine, and the dienoic fatty acyl spermine conjugate N^4,N^9-dilinoleoyl spermine as synthetic lipopolyamines aimed at more efficient NVGT.

DNA condensation was studied using an EthBr fluorescence-quenching assay (λ_{ex} = 260 nm, λ_{em} = 600 nm) (5). N^4,N^9-Dilinoleoyl spermine induced a marked decrease in intercalated EthBr fluorescence intensity from 100 % (EthBr-DNA complex without cationic lipid) reduced to 10 % at N/P charge ratio of 1.5. Light scattering by condensed DNA nanoparticles was detected at λ = 320 nm and a maximum (100 %) relative apparent absorption (UV scattered light) at N/P charge ratio of about 1.5. The transfection efficiencies of the synthesized lipopolyamines were studied in primary skin cells (FEK4 and FCP cells) and in an immortalized (HeLa derived HtTA) cancer cell line using pEGFP as the reporter macromolecule with its fluorescent imidazolidinone moiety analysed by FACS. The results revealed high transfection efficiencies with N^4,N^9-dilinoleoyl spermine, typically 85-90 % in primary cell lines, higher values than were obtained with the saturated N^4,N^9-distearoyl spermine (about 25-40 %) or with N^4,N^9-dioleoyl spermine (about 60-75 %).

The cytotoxicity of these compounds was studied in both primary skin and immortalised cancer cell lines using an MTT assay. N^4,N^9-Dilinoleoyl spermine achieves (about 70 %) viability at N/P charge ratio 5.5. These transfection and cell viability results were compared with non-liposomal cationic lipid Transfectam® that achieved about 46-79 % transfection and 75 % cell viability in the various cell lines at N/P charge ratio 5.9 (cf Fig. 31). Naked (uncomplexed circular) pEGFP DNA typically gave 1-2 % transfection of these cell lines. The results show that an increase in the number of double bonds in the long C18 fatty chain of the cationic lipid improves the ability of the non-viral vector to deliver the DNA payload to the cultured cells.

Condensation of DNA into nanoparticles is a way to decrease the size of the delivered gene in order to facilitate cellular membrane entry by endocytosis and subsequent trafficking to the nucleus. The characterization of particle size by dynamic light scattering revealed that the average particle sizes of both N^4,N^9-dilinoleoyl spermine and Transfectam® were considerably smaller (71 and 62 nm, respectively) than that of N^4,N^9-distearoyl spermine and N^4,N^9-dioleoyl spermine that show increased average particle size of 217 and 366 nm, respectively.

Zeta potential is an important parameter helping to predict the stability of the formulation as well as the ability of the positively charged particles to interact with cell membranes. ζ-Potential results revealed that the surface charge was +43 and +32 mV for N^4,N^9-dilinoleoyl spermine (at N/P charge ratio 5.5) and N^4,N^9-dioleoyl spermine (charge ratio 2.5) lipoplexes, respectively. In addition, gel electrophoresis results revealed that all four spermine conjugates (including Transfectam®) were able to condense pEGFP DNA and completely inhibit the migration of the circular plasmid DNA from lipoplexes in the agarose gel at their respective charge ratio of transfection.

The results show that an increase in the number of double bonds in the long C18 fatty chain of the cationic lipid improves the ability of the non-viral vector to deliver the DNA payload to cultured cells. N^4,N^9-Dilinoleoyl spermine shows high efficiency in DNA condensation and was the most efficient non-viral gene carrier among our investigated lipospermines.

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Appendix

List of Publications

1. I. S. Blagbrough, N. Adjimatera, O. A. A. Ahmed, A. P. Neal, and C. Pourzand. Spermine and lipopolyamines as gene delivery agents. In D. J. Beadle, I. R. Mellor, and P. N. R. Usherwood (eds), *Neurotox '03: Neurotoxicological targets from functional genomics and proteomics,* SCI, London, 2004, pp. 147-159.

2. O. A. A. Ahmed, N. Adjimatera, C. Pourzand, and I. S. Blagbrough. N^4,N^9-Dioleoyl spermine is a novel nonviral lipopolyamine vector for plasmid DNA formulation. *Pharm. Res.* **22**:972-980 (2005).

3. O. A. A. Ahmed, C. Pourzand, and I. S. Blagbrough. Varying the unsaturation in N^4,N^9-dioctadecanoyl spermines: nonviral lipopolyamine vectors for more efficient plasmid DNA formulation. *Pharm. Res.* **23**:31-40 (2006).

List of Presentations

1. **Non-viral Formulation of DNA Using N^2, N^3-Dioleoyl Spermine, (POSTER)**
   British Pharmaceutical Conference, Royal Pharmaceutical Society of Great Britain, Theme: Medicines: from cell to society, Manchester international convention centre, Manchester, UK, September 27th–29th, S-31, (BPC 2004).

2. **Varying the Unsaturation in C18-Lipid Moieties of Spermine-Based Cationic Lipids for More Efficient Non-Viral Gene Delivery, (POSTER)**
   British Pharmaceutical Conference, Royal Pharmaceutical Society of Great Britain, Theme: A common vision for health: Linking science with practice, Manchester, UK, September 26th–28th, S-52, (BPC 2005).

3. **N^4,N^9-Dimyristoyl Spermine is a Non-Viral Cationic Lipid Vector for Plasmid DNA Formulation, (POSTER)**
   British Pharmaceutical Conference, Royal Pharmaceutical Society of Great Britain, Theme: A common vision for health: Linking science with practice, Manchester, UK, September 26th–28th, S-52, (BPC 2005).

4. **N^4,N^9-Dimyristoyl spermine is a non-viral cationic lipid vector for plasmid DNA formulation, (POSTER)**
   Eurpean Federation for Pharmaceutical Sciences, Theme: Optimising Drug Delivery and Formulation, Evaluation of Drug Delivery Systems: Issues and Perspectives, Versailles, France, November 20th–23rd, 157, (EUFEPS 2005).

5. **Efficient non-viral gene delivery using unsaturated C18-lipid conjugates of spermine, (POSTER)**
   Eurpean Federation for Pharmaceutical Sciences, Theme: Optimising Drug Delivery and Formulation, Evaluation of Drug Delivery Systems: Issues and Perspectives, Versailles, France, November 20th–23rd, 156, (EUFEPS 2005).

6. **Condensation and non-viral gene delivery to primary cells by designed lipopolyamines, (ORAL)**
   Royal Society of Chemistry Bio-Organic Subject Group, Postgraduate meeting, School of Chemistry, University of Southampton, UK, December 14th (RSC Bioorg 2005).

7. **Varying the Unsaturation in C18-Lipid Moieties of Spermine-Based Cationic Lipids for More Efficient Non-Viral Gene Delivery, (POSTER)**
   5th World Meeting on Pharmaceutics Biopharmaceutics and Pharmaceutical Technology, Theme: Approaching New Interfaces Geneva, Switzerland, March 27th-30th, 50, 2006.

8. **N^4,N^9-Dimyristoyl spermine is a non-viral cationic lipid vector for plasmid DNA formulation, (POSTER)**
   5th World Meeting on Pharmaceutics Biopharmaceutics and Pharmaceutical Technology, Theme: Approaching New Interfaces Geneva, Switzerland, March 27th-30th, 50, 2006.

9. **Formulation and delivery of p-EGFP DNA condensed by a synthetic lipospermine, (POSTER)**
   British Pharmaceutical Conference, Royal Pharmaceutical Society of Great Britain, Theme: Personalised medicine in healthcare, Manchester, UK, September 4th–6th (BPC 2006).

Neurotox'03: Neurotoxicological Targets from Functional Genomics and Proteomics
D.J. Beadle, I.R. Mellor & P.N.R. Usherwood, editors

CHAPTER 16

SPERMINE AND LIPOPOLYAMINES AS GENE DELIVERY AGENTS

I.S. BLAGBROUGH, N. ADJIMATERA, O.A.A. AHMED, A.P. NEAL and C. POURZAND

Department of Pharmacy and Pharmacology, University of Bath, Bath, BA2 7AY, U.K.

1. INTRODUCTION TO SPERMINE AND LIPOPOLYAMINES

Polyamines are polycationic at physiological pH and play a variety of important biological roles. Many mammalian cells also possess an active polyamine uptake system, although little is known about its function.[1] Spermine (Fig. 1i) (3.4.3 methylene count between the amine groups) and spermidine (Fig. 1ii) (4.3 methylene spacings) were first discovered in the nuclei of sperm, where these polycations help histone proteins to package DNA by charge neutralization of the phosphate anions along the DNA backbone. Most cells use histone proteins and polyamines to condense DNA in the nucleus. The histones found in chromatin are categorized as core histone (subunit H2A, H2B, H3, H4) (Fig. 1) and linker histone (H1), forming an octomer (108kDa) that binds to DNA. Lysine (Lys, K) and arginine (Arg, R), the most important of the positively charged amino acids, are found in significant sequences along most histone proteins. Lys has its $(CH_2)_4NH_3^+$ (basic) side-chain, and Arg is the naturally occurring (mammalian) amino acid containing a guanidine functional group, the most basic functional group in biological chemistry. Thus, the side-chains of these amino acids (together with ornithine and histidine) can be positively charged in the cell. The pK_as of Lys and Arg (side-chains) are 10.5 and 12.5 respectively. DNA-histone complexes are dissociated on treatment with acids or dilute salt solutions.[2] This is evidence of ionic and non-covalent interactions; the phosphate anions are titrated by the ammonium cations. Of course, there also has to be some consideration of spermine (i) and spermidine (ii) and their conjugates as neurotoxins; such neurotoxication can be due to high levels of free polyamines, or similar effects achieved by polyamine amides in the mammalian brain by mechanisms involving glutamate receptors. This latter process parallels the modes of action of the spider and wasp polyamine amide (invertebrate) neurotoxins.

2. OVERVIEW OF NON-VIRAL GENE THERAPY (NVGT)

Gene therapy is a new treatment strategy for some difficult-to-cure diseases, introducing a therapeutic gene into body cells and aiming for desired gene expression. The therapeutic outcome is not only limited to missing proteins in patients (such as a chloride ion channel in

the lungs of patients with cystic fibrosis), but also an inhibitory protein signal to stop the progression of disease, especially as a potential treatment for certain cancers.[4] The design and synthesis of efficient DNA delivery vectors are major research areas in non-viral gene therapy (NVGT). Lentiviral and other viral vectors show efficient transfection, however, there is the possibility of severe toxicity from the viral genome, as well as immunogenicity, and there is the drawback of a limited DNA payload. Naked DNA is used even in current clinical trials. NVGT vectors have been produced to retain or to improve upon the performance of viral vectors and to provide a safe alternative for clinical gene therapy.[4] Recently, Felgner *et al.* reported a system of NVGT nomenclature guidelines to aid in NVGT research, dividing the vectors into the two major synthetic gene delivery systems: lipoplex (cationic lipid-nucleic acid complex) e.g. lipopolyamines, cationic liposomes, and polyplex (cationic polymer-nucleic acid complex) e.g. polyethylenimine (PEI), poly-L-lysine (PLL) etc.[5] Gene therapy is a complex drug (DNA) delivery strategy which must overcome intracellular barriers. The contributions from these barriers in blocking efficient NVGT are not well understood. We are aiming to understand (Fig. 2) and, thereby, to design a significant improvement over current transfection yields.

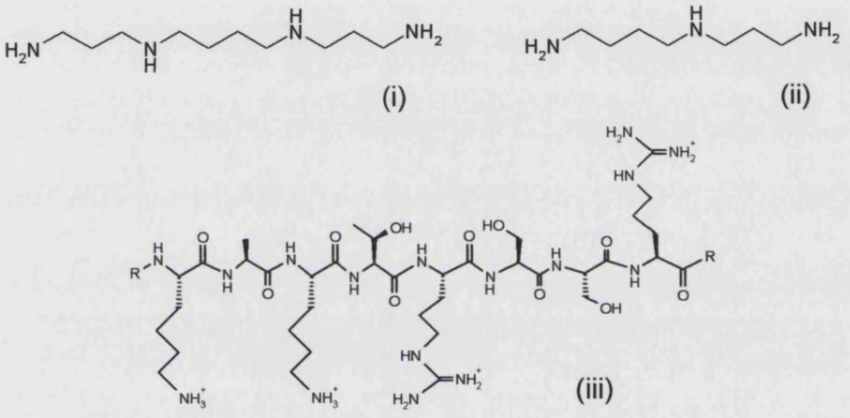

Figure 1 *Spermine (i), spermidine (ii) and histone 2A (iii) K(13)AKTRSSR(20) (underlined amino acids representing some DNA contact sites of histone 2A are shown).*[3]

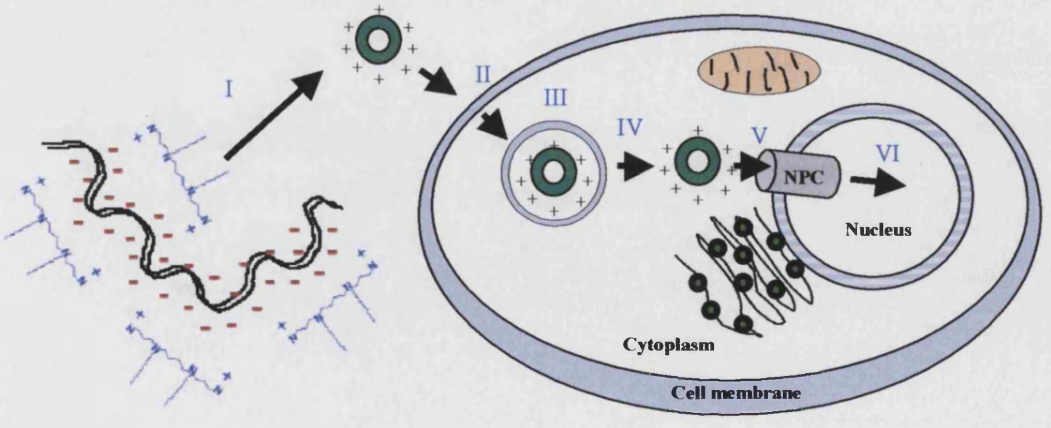

Figure 2 *Barriers to NVGT (I – DNA condensation and particle formation, II – endocytosis, III – endosome escape, IV - nuclear localization, V - nuclear entry and VI - gene expression).*

Negatively charged DNA (due to the phosphate anions), when neutralized by salt formation with positively charged lipopolyamine vectors, results in condensed nanometer-sized particles typically 50-150nm in outer diameter (Fig. 4). Nanoparticles, toroidal in shape, were observed in polyamine-induced DNA condensation.[6,7] This DNA compaction facilitates its stability in extracellular compartments, cellular uptake, and other intracellular process such as nuclear entry.[7,8] The electrostatic interactions between the surface of DNA particles and cell membranes is expected to be an initial process leading to internalization by endocytosis of DNA nano-complexes, with the involvement of clathrin-coated pits on the cell membrane.[9,10] It has been shown that endosome escape of the DNA is one major critical barrier to efficient transfection.[8] Early endosomes are formed by the inversed cell membrane upon DNA particles endocytosis, which are eventually degraded into late endosomes by internalization into lysosome vesicles. The "proton sponge" hypothesis has been proposed for endosome escape of NVGT cationic polymer. The protons are pumped into endosomes (at pH 7.4) by V-ATPase proton pump at their membrane, while polycations (the pK_a of a primary amine is around 10.5)[11] work as a pH buffer material. This results in an increased proton/water flux and lower pH at 5.5. Membrane disruption by swelling and finally osmotic lysis allows DNA complexes to escape from these vesicles prior to enzymatic degradation. Alternatively, the mechanism of lipid membrane mixing between endosomal membrane and lipidic gene carrier has also been hypothesized as an alternative means by which cationic lipids/liposomes might facilitate the endosome escape process, although this process is not well understood.[8,12,13]

Also, the mechanism of DNA nuclear translocation is not well understood. However, the transport through nuclear pores has been considered as the most likely possible route in conjunction with help from nuclear localization signals (NLS). NLS are peptides that help DNA complexes (which are typically bigger than 50kDa and cannot pass through the nuclear pore complex) to reach the nucleus. NLS binds with the α-subunit of the cytosolic importin receptor, then the β-subunit of the importin binds to the nuclear pore complex with the help of GTP binding protein (called Ran). The β-subunit will be retained at the inner face of pore complex.[14] Based on amino acid sequence analysis of NLS such as SV40 T-antigen, HIV-tat protein etc., positively charged amino acids are also found as their unique characteristic.[14] The nuclear envelope contains double phospholipid layers, with the membrane space directly connecting to the endoplasmic reticulum lumen. The inner and outer membranes join together at the nuclear pore complex (NPC). The NPC is a large structure with an assembly of eight spokes attached to rings on the cytoplasmic and nuclear sides of the nuclear envelope, accordingly named "the cytoplasmic ring" and "the nuclear ring", with a formed central channel approximately 40nm in diameter for the transportation of large particles across the nuclear membrane. The pore diameter is 120nm and the molecular mass is about 125 million Da. The small molecules (size of 9nm diameter, size less than 50kDa), for example, ions, metabolites and small proteins, use passive diffusion to get in and out of the nucleus freely. Bigger molecules, including DNA, are transported by an energy-dependent mechanism through this NPC. The selective transport of these macromolecules to and from the nucleus requires a NLS to direct their traffic through the NPC.[14]

3. SPERMINE MEDIATED DNA CONDENSATION AND PARTICLE FORMATION

Spermine (i) is a natural biomolecule involved in cell growth and differentiation control. *In vitro* studies have shown that the binding of spermine to DNA induces structural changes in DNA. The spermine cations neutralize the negatively charged phosphodiester groups of DNA, initiating the DNA helix's axis bending resulting in DNA condensation. This gene

packing enables efficient gene therapy by non-viral vectors. The results show that the nanoparticle (50-150nm) is able to go across the cell membrane and lead to the gene expression of delivered DNA.[7,15] The ethidium bromide (EthBr) assay was used to measure DNA condensation. EthBr is a cationic dye (Fig. 3) that intercalates between DNA base pairs. This intercalation increases the fluorescence yield of EthBr (excitation 546nm, emission 595nm). Many compounds that bind to DNA, including spermine, can displace EthBr from the EthBr-DNA complex. The EthBr displacement by these chemicals is related to DNA bases as well as environmental conditions (e.g. buffer composition, ionic strength). So, EthBr fluorescence quenching can be used to measure the DNA-polyamine binding efficiently.[15,16]

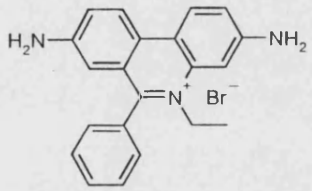

Figure 3 *Ethidium bromide.*

The EthBr assay method is a modified literature protocol of Cain and co-workers[17], regarded as an "exclusion assay" requiring the pre-forming of DNA and DNA binding drugs, before EthBr is added. The excitation wavelength used is 546nm, which is based on the excitation of EthBr. In 2000, the modified reproducible EthBr "displacement assay" was reported by Geall and Blagbrough.[16] The excitation wavelength was optimized at 260nm, so (only) intercalated EthBr is indirectly excited by energy transfer from the DNA. The proposed protocol also allows the experiment to be run without pre-complexing DNA and its binding molecule. This method is named as "displacement assay".[16] Additionally, the EthBr-DNA complex formation restricts the internal rotational freedom of EthBr. The fluorescence polarization is calculated from the emission intensity from polarizer and analyzer in a spectrofluorimeter. The polarization increase is observed when DNA is condensed due to the limited movement of EthBr. The measurement of steady-state fluorescence polarization can be used to indicate the EthBr-DNA complex conformation upon binding of spermine.[15]

In addition to the intercalating assay using EthBr, the UV absorbance at over 300nm (e.g. 320nm) was useful in confirming the formation of nanoparticles. The double helix DNA was bound by polyamines and formed the nanoparticles which scatter the light resulting in the UV absorbance increase above 300nm.[18] So, light scattering (LS) is being measured rather than (apparent) UV absorption. Some precipitation of the DNA may be visible, but it does not increase the absorption above 300nm. However, the DNA concentration used in this assay was in a 10-fold excess compared to the EthBr assay given the low sensitivity of this experiment and the lack of fluorescent indicator.[11]

DNA used in gene therapy research is normally prepared as plasmids, usually with a protein marker that can be followed in the gene expression quantification. pEGFP[19] (4.7 kilobasepairs) is the plasmid encoding for enhanced green fluorescent protein, under CMV (cytomegalovirus) promoter, obtained from Clontech. Successfully transfected cells with pEGFP shows the distinct fluorescence detectable by a Fluorescent Activated Cell Sorter (FACS). Modified firefly (*Photinus pyralis*) luciferase pGL3 control vector[20] (5.2 kilobasepairs), with SV-40 promoter, was from Promega. In this luciferase assay, luciferin was added to transfected cells that underwent oxidation and generated the yellow light, measured by a luminometer.

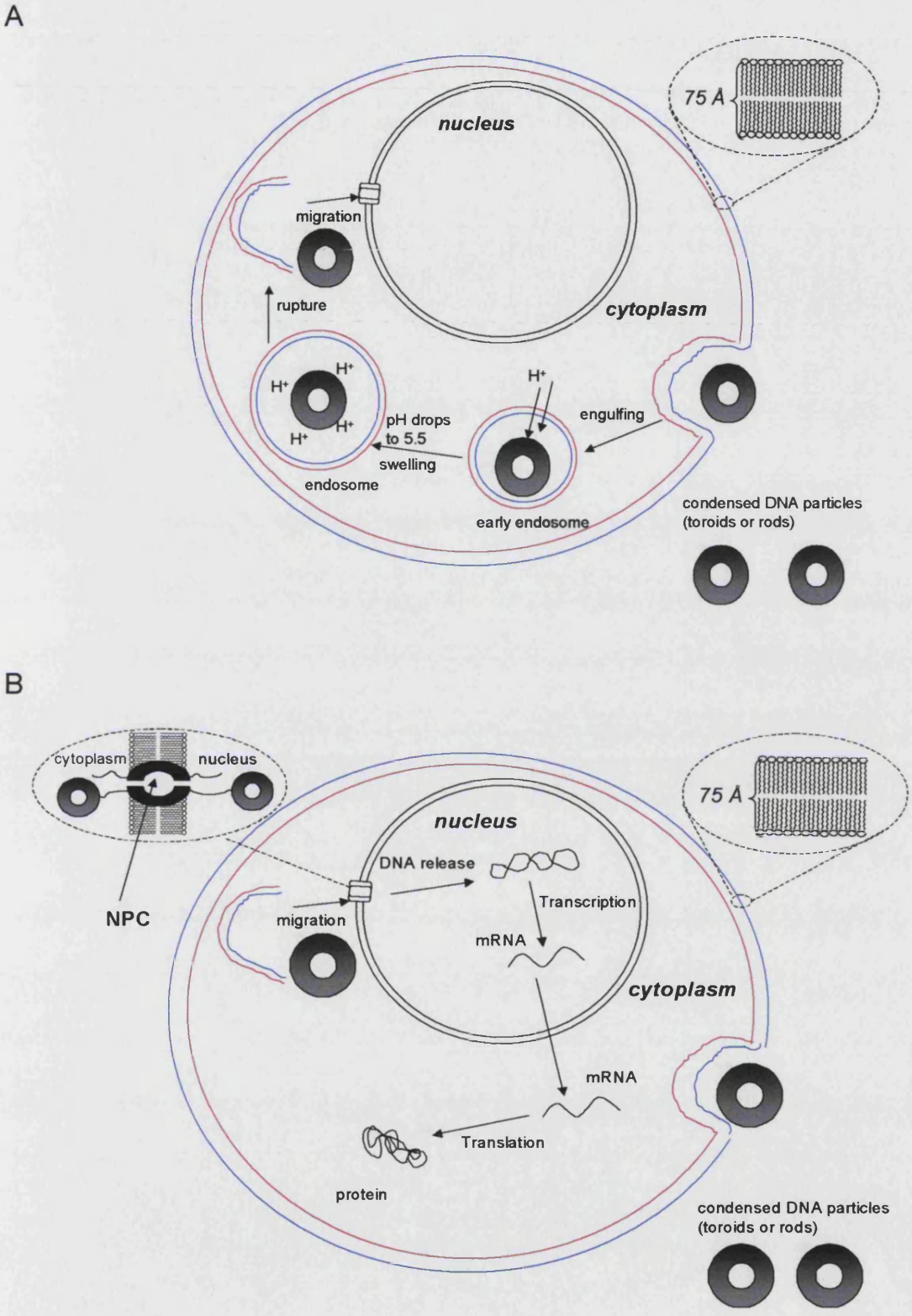

Figure 4 *Detailed NVGT mechanism. A: endocytosis process. B: nuclear entry and gene expression process.*

pEGFP and pGL3 were condensed by spermine to about 50%, measured using the EthBr assay (Fig. 5). Particle formation was also confirmed by the LS assay, with significantly increased apparent absorbance at an N/P ratio > 3.00. We conclude that (simple) spermine-mediated condensation may provide less compacted particles; also these are only really significant at high N/P ratios. Additionally, the condensation profiles of both plasmids were found to be similar, possibly relative to their similar plasmid sizes. We and others have shown that lipopolyamines are more efficient DNA condensers (though not necessarily NVGT vectors) than these simple polyamines.[21]

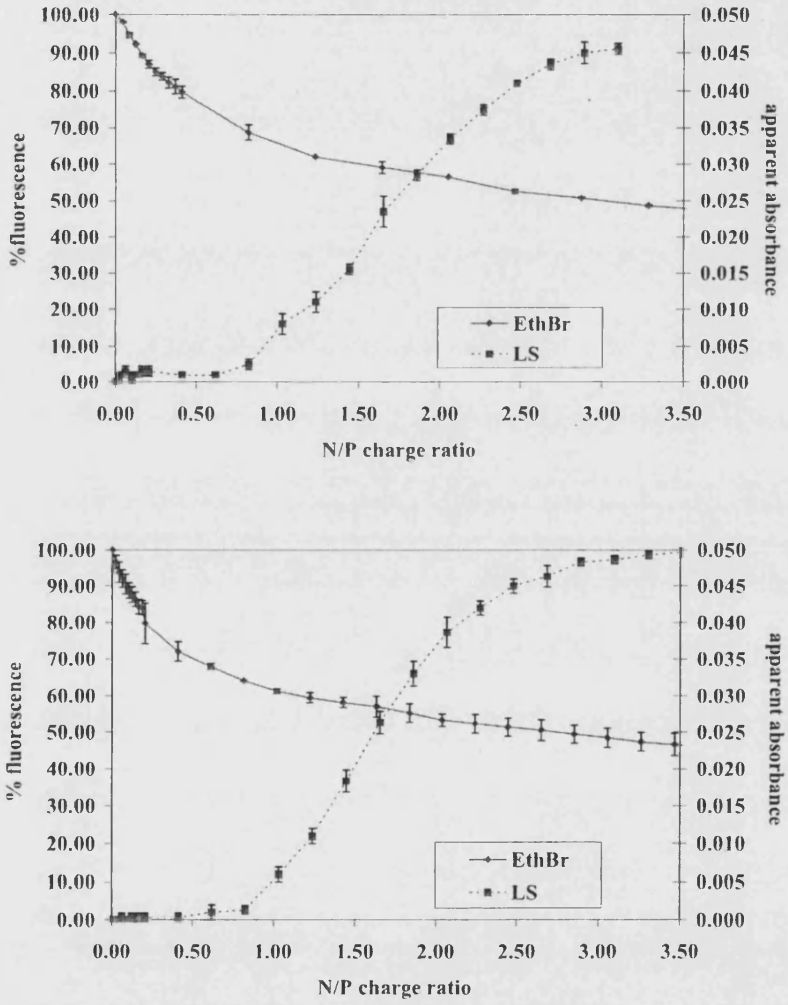

Figure 5 *DNA condensation and light scattering of nanoparticles (above – pEGFP plasmid vs spermine, below – pGL3 vs spermine).*

4. DEVELOPMENT OF POLYCATION VECTORS IN NVGT

A variety of polycations, but nevertheless small molecules compared to histones or DNA, have now been reported for studies in NVGT (Fig. 6). The examples chosen serve to illustrate the current range of molecular diversity within lipopolyamines. From one of the earliest steroid conjugates, DC-Chol containing only one positively charged N-atom, through

Genzyme's GL#67, Behr's Transfectam (DOGS), Scherman and co-workers' RPR-120535 and DOSPA (all containing spermine in different ways), through to vitamin D and modified buckyball (C60) conjugates. The most recent lipopolyamine is KanaChol from Lehn and co-workers.[23]

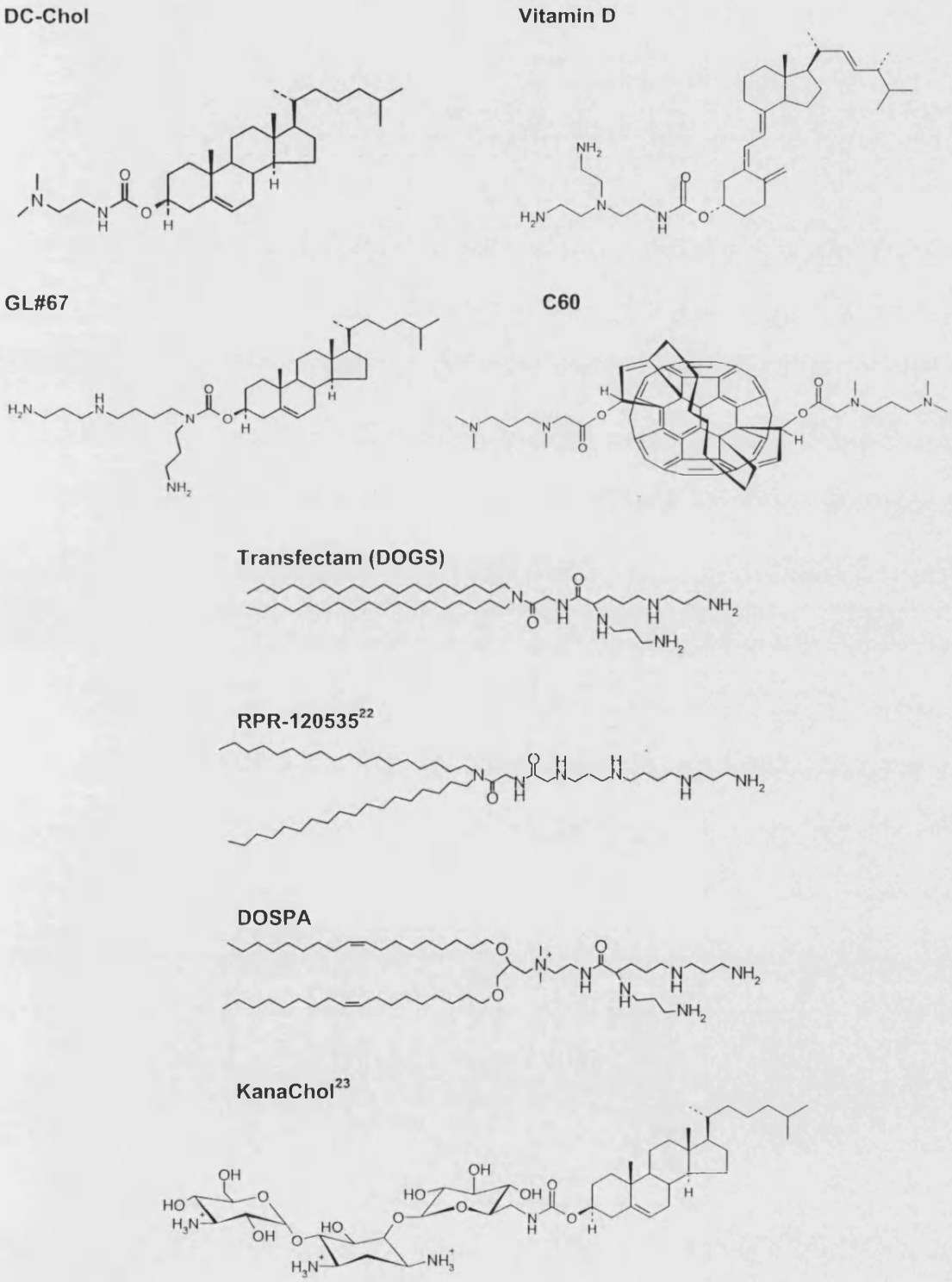

Figure 6 *A selection of lipopolyamine-based NVGT vectors (lipoplex).*[21]

Polymer polycationic NVGT vectors (Fig. 7) for the formation of polyplexes are based upon linear and branched polyethylenimines, PLL, poly-L-arginine and poly-L-histidine. These representative examples demonstrate the distribution of positive charge in the presence of a lipid region, cationic lipids.

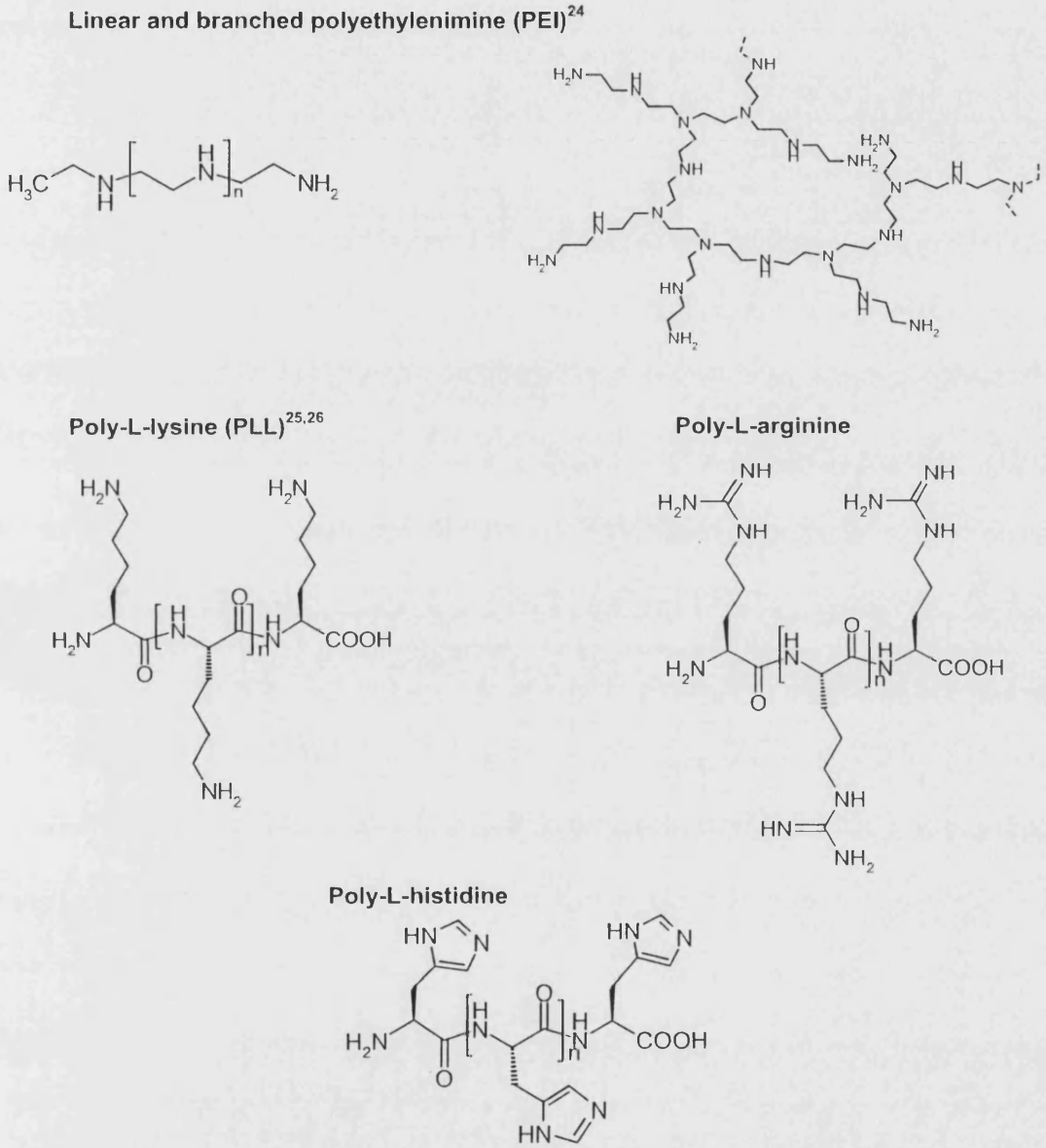

Figure 7 *Polymer polycationic NVGT vectors (polyplex).*

5. PHYSICOCHEMICAL PROPERTIES OF POLYAMINES-BASED DNA CONDENSING AGENTS

The effects of the regiochemical distribution of positive charges along the polyamine moiety in DNA condensing agents were studied by Geall *et al.*[11,27] DNA condensation is dependent upon

three characteristic properties of polyamines: the number of positive charges, the regiochemical distribution of charges (determined by the pK_a of each amino group), and the local salt concentration. From the pK_a investigation and calculation, spermine (i) carries 3.8 charges and its cholesterol conjugate has a positive charge distribution similar to that found in spermidine (ii) (4.3) (Fig. 8). Such spermidine mimics (lipospermidines) are still effective as DNA condensing agents, significantly more so than spermidine itself, which is but a weak DNA condensing agent.

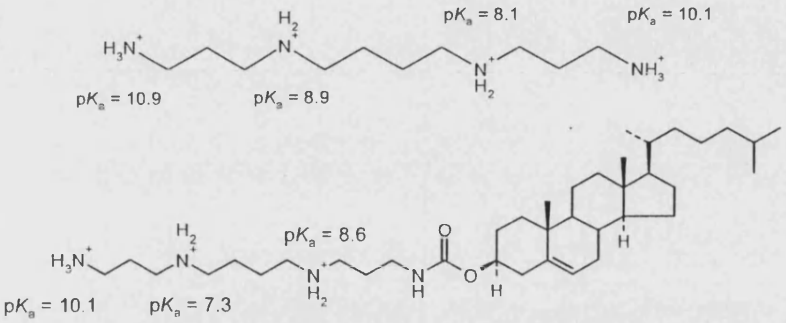

Figure 8 *p*K$_a$ *of spermine and a cholesteryl lipopolyamine.*[11]

We have designed and prepared lipopolyamines (Fig 9) and monitored their efficiency in DNA condensation into particles by LS and their salt-dependent binding affinity for DNA by fluorescence quenching.[11,27] Lipopolyamines were prepared from cholesterol, lithocholic and cholic acids (5β-cholanes) by acylation of tri-Boc-protected tetra-amines spermine and thermine. These ligands are polyammonium ions at physiological pH.[28]

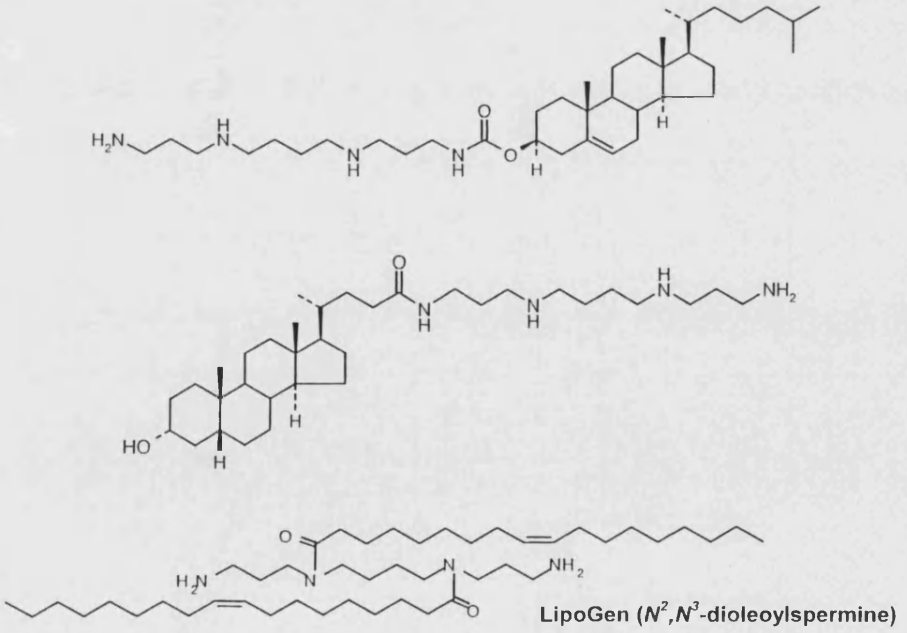

Figure 9 *Lipopolyamines based on cholesterol*[11]*, bile acids*[28] *and fatty acids.*

LipoGen (from InvivoGen) is an example of a lipopolyamine with two oleoyl groups at N^2 and N^3 of spermine, therefore with only two positively charged nitrogens. Modifying the lipophilicity of spermine (3.4.3) led to efficient DNA condensation (15% residual fluorescence at N/P charge ratio 2.5) comparing to the tetracationic spermine (50% at charge ratio more than 3.50) (Fig 10). According to Vijayanathan *et al.*[29] the λ–phage DNA condensed particles (by spermine) are in the size range 50-100 nm. Lipospermine was shown to condense pSfiSVneo, pSifiSV19 and pCISfi-γIFN DNA to smaller particle sizes at 50-70nm.[30] The efficient condensation of DNA by lipospermine over spermine, resulting in smaller sized particles, may promote more effective transfection.

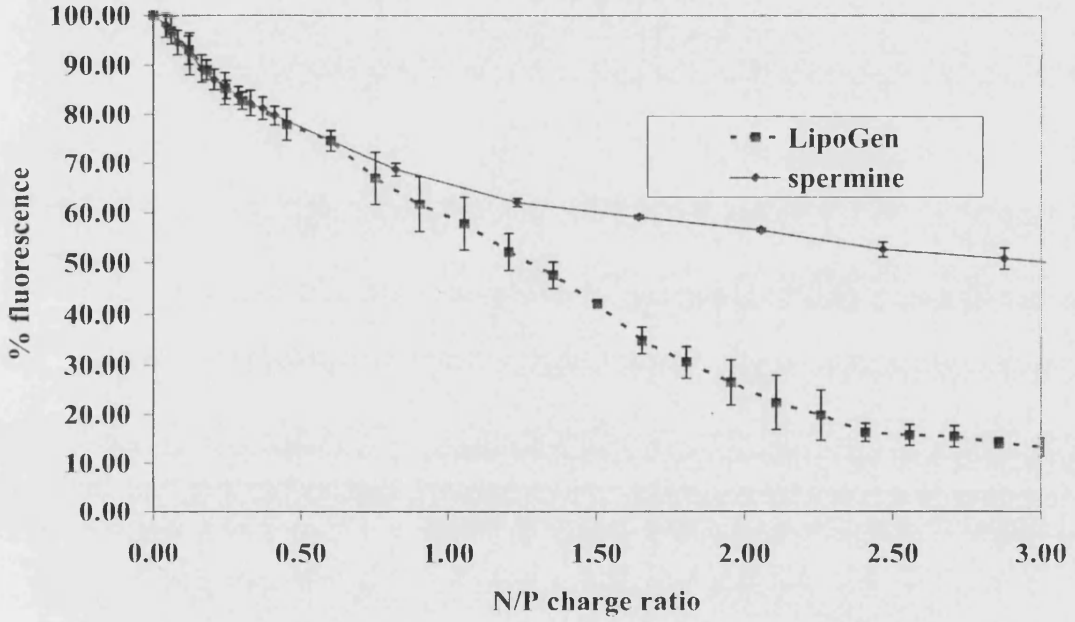

Figure 10 *pEGFP condensation by LipoGen vs spermine.*

6. TRANSFECTION IN HUMAN SKIN FIBROBLAST CELLS (FEK4)

LipoGen (Invivogen) is a lipospermine with two oleoyl groups at N^2 and N^3 of spermine (i) and, therefore, only two positively charged primary amines. It was prepared as a non-liposomal formulation. The lipophilic modification aims to facilitate the transfection process (e.g. potentially through enhanced DNA condensation, cell entry, endosomal escape). This lipophilic modification of the spermine structure resulted in a more efficient pEGFP DNA condensation (15% residual fluorescence, in the EthBr assay, at N/P charge ratio 2.5) compared to tetracationic spermine (50% at N/P charge ratio 3.0). The *in vivo* transfection of pEGFP (2μg/well) with LipoGen was carried out using FEK4[31] (2.5×10^4 cells/well at 50% confluence), incubated for 4h, then the transfection was stopped by removal of the DNA complex and replacement with foetal-calf serum containing media; FACS analysis was performed 48h post-transfection. By using pEGFP as delivered DNA, the EGFP chromophore (a substituted 4-hydroxybenzylidene imidazolidinone) was detected in successfully transfected cells by FACS (fluorescent-activated cell sorting). The EGFP amino acid sequence L(64)TYGV(68) transcribed from pEGFP was cyclised in the post-translational

steps (at amino acids 65-67) forming an EGFP fluorophore with λ ex = 488nm (red-shifted from wild GFP protein) and λ em = 507nm.[19] Lipofectin (DOTMA/DOPE = 1:1 w/w, Invitrogen), the commonly used transfection liposomal reagent containing cationic lipid (with one positive charge) and helper lipid (DOPE), was also used in this experiment for a comparison. The fluorescent cell counts observed in all N/P ratios were higher in LipoGen-mediated transfection. Thus, the lipid moiety in lipospermine is playing an important role in *in vitro* transfection. The optimal charge ratio for FEK4 transfection with pEGFP-LipoGen complex is around 2.5, which corresponds to the optimal DNA condensation N/P ratio (from the EthBr assay). However, from Fig. 11 it appears that increased condensation leads to higher transfection efficacy. The high N/P ratio (more than 5.0) results in less efficient gene delivery overall. A similar relationship between N/P charge ratio and transfection efficiency was also observed in Lipofectin-mediated transfection.

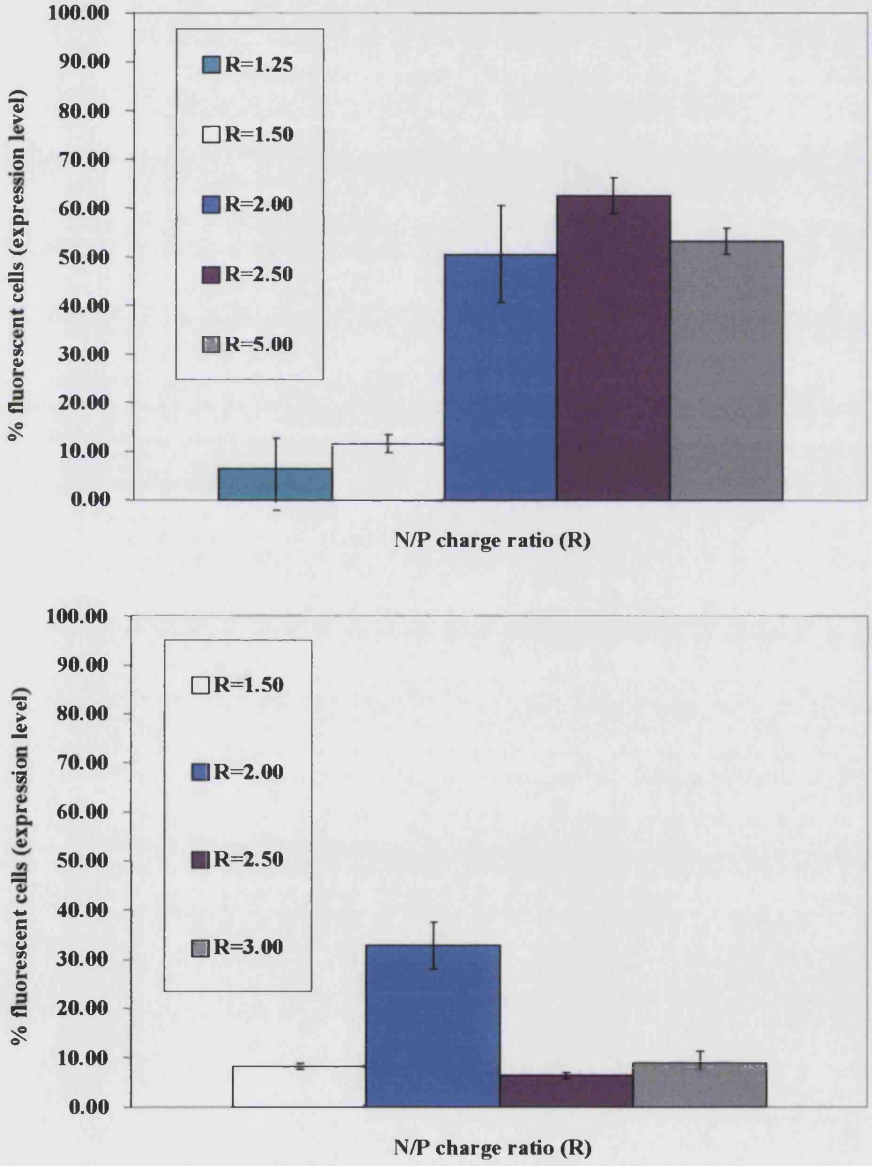

Figure 11 *FEK4 Transfection of pEGFP complexed by LipoGen (above) and Lipofectin (below).*

7. CONCLUSIONS

Our research has advanced neurotoxic polyamine amides from spider and wasp natural products,[32] with their exquisite sensitivity in modulating glutamate receptors, from pharmacological tools towards therapeutic neurochemistry,[33] thereby highlighting their potential as therapeutic agents.[34] So, whilst polyamines and polyamine amides are potent and selective receptor probes for a variety of voltage- and especially for ligand-gated cation channels,[35] they also are now seen to have potential as synthetic vectors and can be exploited in NVGT.[21,35] Using our designed steroidal lipopolyamine probes (Fig. 9), we are studying DNA-lipopolyamine complexes with respect to their formation by DNA condensation. Comparable DNA-binding efficiency to that of the unlabelled lipopolyamines and robust fluorescent spectral properties across the varying cellular pH range are desirable properties in fluorescent ligands, but this requires experimental verification. Have we designed and prepared fluorophore-substituted lipopolyamines or lipopolyamine-substituted fluorophores? Only time and experimental results will tell. However, we have achieved a controlled chain extension of suitably protected polyamines using reductive alkylation to mimic spermine (i) rather than spermidine (ii) in the target molecules.[21] A practical method for the efficient hydroboration of cholesteryl carbamates has allowed us to prepare our designed *trans*-AB steroidal lipopolyamines, together with the corresponding *cis*-AB ring junction as the minor product of hydroboration or from naturally occurring bile acids.[21] We are able to introduce fluorophores of choice by our Fmoc-chemistry route. The design in our versatile synthetic route to Fmoc protected aminoesters of cholesteryl carbamate and lithocholic acid polyamine amides allows a range of selected fluorophores to be readily incorporated. The design and synthesis of these novel fluorescent lipopolyamines allows us to study the intracellular events during transfection. As well as our studies on lipopolyamine-mediated DNA condensation, we are optimising these probes to employ them monitoring the intracellular hurdles to NVGT. At present, a poor understanding of the molecular mechanisms of action of non-viral vectors remains an important, but unresolved issue. With a greater knowledge of these mechanisms, new (possibly spermine based) lipopolyamine vectors with improved transfection efficiency can be rationally designed and used as gene delivery agents *in vivo*.

Acknowledgements

We thank the Embassy of the Arab Republic of Egypt and the EPSRC for PhD studentships to O.A.A.A. and A.P.N. respectively. We acknowledge the support of Universities U.K. through an ORS award to N.A. We are also grateful to Prof R.M. Tyrrell (University of Bath) for the FEK4 cell line.

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Pharmaceutical Research, Vol. 22, No. 6, June 2005 (© 2005)
DOI: 10.1007/s11095-005-4592-1

Research Paper

N^4,N^9-Dioleoyl Spermine Is a Novel Nonviral Lipopolyamine Vector for Plasmid DNA Formulation

Osama A. A. Ahmed,[1] Noppadon Adjimatera,[1] Charareh Pourzand,[1] and Ian S. Blagbrough[1,2]

Received November 28, 2004; accepted February 15, 2005

Purpose. To study the effect of synthesized N^4,N^9-dioleoyl spermine on DNA condensation and then measure its transfection efficiency in cell culture.
Methods. The lipopolyamine was synthesized from the naturally occurring polyamine spermine. The ability of this novel compound to condense DNA was studied using ethidium bromide fluorescence quenching and light scattering assays. Transfection efficiency was studied in primary skin cells (FEK4) and in an immortalized cancer cell line (HtTA), and compared with the commercially available transfection formulations Lipofectin and Lipofectamine.
Results. The synthesized N^4,N^9-dioleoyl spermine formula is efficient at condensing calf thymus and circular plasmid DNA and effectively transfects both primary skin cells and cancer cell lines at low charge ratios of (+/− ammonium/phosphate) 2.5.
Conclusions. N^4,N^9-Dioleoyl spermine condenses DNA and achieves high transfection levels in cultured cells.

KEY WORDS: FEK4; gene delivery; lipopolyamine; N^4,N^9-dioleoyl spermine; transfection.

INTRODUCTION

It is widely believed that gene therapy will become an efficient medicine for the treatment of diseases such as cancer, cystic fibrosis and for vaccination. The essential requirements for gene delivery are the transport of DNA through the cell membrane and ultimately to the nucleus. The design of an efficient formula for the delivery of genetic material requires a detailed understanding of the mechanism of gene delivery to the nucleus. Different strategies have been used for the delivery of genetic material into target cells, classified as viral or non-viral delivery systems (1–3). Viral delivery systems depend on the development of genetically-modified viruses to utilize their capability of efficiently delivering DNA into cells without their pathogenic characteristics (3). Although high efficiency is achieved by viral vectors, there are concerns about their use which include: a limit in the size of the DNA delivered (the "payload"), endogenous viral recombination, unexpected anti-vector immune response, and oncogene activation (4–7). Since the design and formulation of Lipofectin by Felgner and co-workers, reported in 1987 (8), the focus on nonviral vectors for DNA delivery has shown a remarkable increase worldwide (9–15).

Efficient nonviral formulation should be able to deliver safely the required DNA across the various cellular barriers to the nucleus. These barriers that hinder the delivery of DNA to its physical site of action (the nucleus) have been summarized in Fig. 1. They include complex formation between the DNA and cationic lipid or polymer that leads to condensation of DNA into nanoparticles. Cell-membrane entry is thought to be mediated by cationic substances, which interact with the DNA payload, and can then cause adsorptive endocytosis and internalization of the complex. Also, the lipid moiety in cationic lipids interacts with the phospholipid bilayer of the cell membrane that facilitates cell entry. The internalized material is fused with early endosome. That leads to sorting to the late endosomal compartment, at this stage the DNA complex should escape the endosomal vesicle before the later stage of the lysosome where the DNA will be degraded. After endosomal escape, the DNA (either complexed or dissociated from the condensing agent) should find its way to the nucleus and cross the nuclear membrane which is thought to occur through the nuclear pore complex (NPC) or by direct association with the chromatin during mitosis. After nuclear entry, the payload DNA should successfully be able to give the desired protein through the processes of transcription and translation.

For drug formulators, it is difficult to deliver a drug molecule of 3.3 kDa molecular weight carrying 10 negative charges, but in the case of the (prodrug) DNA, a 5 kbp

[1] Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, United Kingdom.
[2] To whom correspondence should be addressed. (e-mail: prsisb@bath.ac.uk)

ABBREVIATIONS: DCC, dicyclohexylcarbodiimide; DOGS, dioctadecylamidoglycylspermine; DOSPER, 1,3-dioleoyloxy-2-(6-carboxyspermine; EGFP, enhanced green fluorescent protein; EMEM, Earle's minimal essential medium; EthBr, ethidium bromide; FCS, fetal calf serum; HOBt, hydroxybenzotriazole; NPC, nuclear pore complex; PEI, polyethylenimine; PLL, poly-L-lysine.

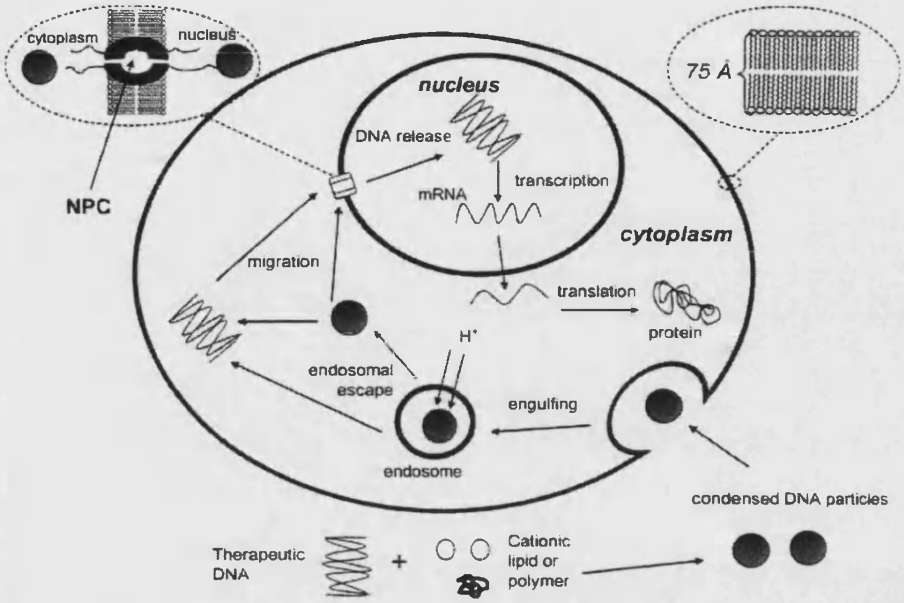

Fig. 1. Steps in the process of nonviral gene therapy by endocytosis showing the barriers for DNA nanoparticles, from the formation of the DNA-polycation complex (condensed DNA particles) to protein synthesis.

plasmid has a molecular weight of about 3.3 megadaltons and carries 10,000 negative charges. So the first key step in gene formulation is DNA condensation into a nanoparticle form through masking the negative charges of the phosphate backbone which causes alleviation of charge repulsion between remote phosphates on the DNA helix leading to collapse into a more compact structure (2,16). The importance of DNA condensation is attributed to the correlation of the transfection efficiency with the formation of DNA nanoparticles that are essential for the delivery of DNA through the cell membrane (16–20).

Cationic lipids are considered to be the major gene carriers among the non-viral delivery systems. They have the ability to condense DNA into particles that can be readily endocytosed by cultured cells, and facilitate endosomal escape leading to efficient delivery to the nucleus (21). They can be classified as liposomal and non-liposomal nonviral delivery vectors. Liposomal delivery vectors usually contain two types of lipids, a cationic lipid (positively charged amphiphile) for DNA condensation and cellular membrane interaction, and a neutral helper lipid (phospholipid), most use dioleoylphosphatidyl-ethanolamine (DOPE) (Fig. 2) to increase transfection efficiency as it has a membrane fusion promoting ability (8,22,23). Nonliposomal cationic-lipid delivery vectors combine both the characteristics of cationic and helper lipids.

The synthesis of the lipopolyamine dioctadecylamidoglycylspermine (DOGS) by Behr and co-workers (24) as a promising transfecting agent, encouraged several laboratories to focus on the synthesis of novel cationic lipids based on the naturally occurring polyamine spermine, for example, RPR120535 (25) and 1,3-dioleoyloxy-2-(6-carboxyspermine) DOSPER (26) (Fig. 2). The design of a novel lipopolyamine formula for DNA condensation and cellular delivery relies on previous and continuing studies of the structure-activity relationships of DNA binding and condensation by polyamines (16,27–30). Although lipopolyamines are less efficient in comparison with viral vectors, their promising lower toxicity than viral vectors ensures a continuous effort to design novel lipopolyamines with improved transfection efficiency. In this study, we synthesized and formulated a novel lipospermine in which the tetra-amine spermine (the cationic moiety) and dioleoyl chains (the lipophilic moiety) that is reported to improve the transfection efficiency by fusion with cellular membrane (31). These unsaturated chains are linked by amide bonds at the secondary amino groups of spermine to form N^4,N^9-dioleoyl spermine (commercially available as LipoGen) (32). These amide linkers have the advantages of being both biodegradable and less toxic than the ether bonds in DOTMA (33,34). The ability of this synthetic lipopolyamine to condense DNA was studied using ethidium bromide (EthBr) fluorescence quenching and light scattering assays. Transfection efficiency was studied in an immortalized cancer cell line (HtTA), and in primary skin cells (FEK4) for the first time. The difficulties found in efficiently transfecting primary cell lines were largely overcome with this nonliposomal formulation comprising a vector with two covalently bound oleoyl chains. The results are compared with two commercially available (liposomal) transfection formulations, Lipofectin® and Lipofectamine that incorporate such oleoyl or oleyl (C18) chains.

MATERIALS AND METHODS

Materials

Chemicals, including polyamines spermine, polyethylenimine (PEI), and poly-L-lysine (PLL), reagents, solvents, buffers, and DNA were routinely purchased from Sigma-Aldrich, UK, except where indicated. Lipofectin and Lipo-

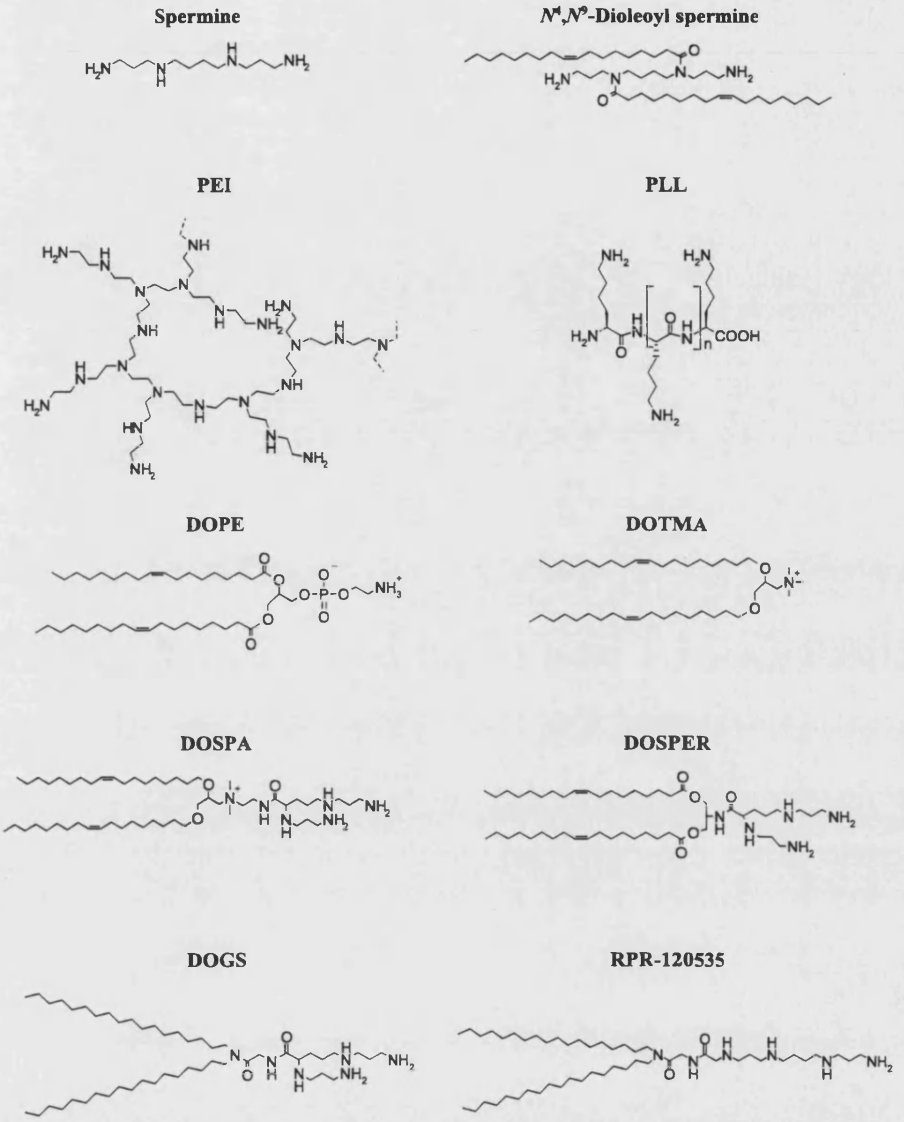

Fig. 2. Chemical structures of spermine, PEI, PLL, DOPE, DOTMA, and spermine based lipopolyamines.

fectamine reagents from Invitrogen (Life Technologies Gibco BRL) and cell cultures materials from Life Technologies (Paisley, Scotland).

Synthesis of N^4,N^9-Dioleoyl Spermine

Spermine was used as the starting material for the synthesis process (35), outlined in Fig. 3. Spermine was protected on the primary amino functional groups with ethyl trifluoroacetate (2.2 eq) in methanol and the reaction mixture was stirred for 18 h at 25°C. The solvent was evaporated to dryness *in vacuo* to form N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane. Dicyclohexylcarbodiimide (DCC, 2.5 eq), 1-hydroxybenzotriazole (HOBt, 0.2 eq) and oleic acid (2.2 eq) were added to the diprotected spermine solution in CH_2Cl_2 and methanol (1:1). The solution was stirred for 18 h at 25°C. The solvent was evaporated to dryness *in vacuo*. The residue was dissolved in CH_2Cl_2 and the solution filtered and evaporated to dryness *in vacuo* to form N^4,N^9-dioleoyl-N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane. For the removal of the di-trifluoroacetyl groups, the tetra-amide was dissolved in methanol and the pH of the solution was increased by saturating with ammonia gas, then it was left (18 h) and evaporated to dryness *in vacuo* to give a residue which was purified over silica gel (CH_2Cl_2-MeOH 5:3 v/v, then CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v) to afford N^4,N^9-dioleoyl spermine R_f 0.3 (CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v).

Amplification and Purification of Plasmid DNA (pEGFP)

DNA plasmid encoding enhanced green fluorescent protein (pEGFP) purchased from Clontech was transformed into *Escherichia coli* JM 109 bacterial strain (Promega). The

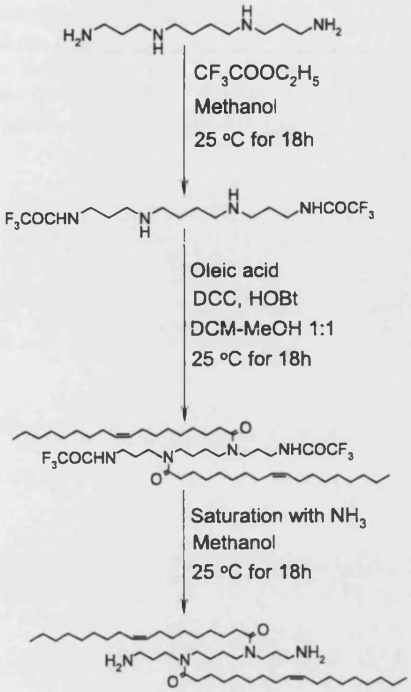

Fig. 3. Synthesis of N^4,N^9-dioleoyl spermine.

transformed cells were grown in larger quantities of Luria-Bertani (LB) broth supplemented with 125 mg/L ampicillin. pEGFP plasmid was produced in large-scale using HiSpeed plasmid purification Maxi kit (Qiagen) according to the manufacturers protocol. DNA yields and purity were determined spectroscopically (OD_{260}/OD_{280} = 1.80 to 1.90 OD, optical density) and by agarose gel (1% agarose) analysis.

Ethidium Bromide Fluorescence Quenching Assay

Each concentration of the DNA stock solutions (approximately 1 μg/μl, 1 ml) was determined spectroscopically (Milton Roy Spectronic 601 spectrometer, 1 cm path length, 3 ml cuvette) (2) and 6 μg (approximately 6 μl) of DNA was diluted to 3 ml with buffer (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) in a glass cuvette stirred with a micro-flea. Immediately prior to analysis, EthBr solution (3 μl, 0.5 mg/ml) was added to the stirring solution and allowed to equilibrate for 10 min. Separately for each polyamine or lipopolyamine (spermine, poly-L-lysine (average molecular weight 9600 Da, PLL 9.6k), polyethylenimine (average molecular weight 2000 Da, PEI 2K), N^4,N^9-dioleoyl spermine, Lipofectin, and Lipofectamine) aliquots (5 μl, according to the ammonium/phosphate (+/−) charge ratio required) were then added to the stirring solution and the fluorescence measured after 1 min equilibration using Perkin-Elmer LS 50B luminescent spectrometer (λ_{excit} = 260 nm and λ_{emiss} = 600 nm with slit width 5 nm) while stirring using an electronic stirrer (Rank Bros. Ltd.) (36). The total polyamine solution added to the DNA solution did not exceed 5% of the total volume of the solution, so no correction was made for sample dilution. The fluorescence was expressed as the percentage of the maximum fluorescence when EthBr was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free EthBr in solution.

Light Scattering Assay

DNA (60 μg, 60 μl of 1 mg/ml solution) was diluted to 3 ml with HEPES buffer (2 mM HEPES, 20 mM NaCl, 10 μM EDTA, pH 7.4) in a cuvette with a micro-flea, and the concentration determined spectroscopically (Milton Roy Spectronic 601 spectrometer, 1 cm path length, 3 ml cuvette). Then aliquots (5 μl, according to the ammonium/phosphate (+/−) charge ratio) of the tested polyamines were then added to the stirring solution and the absorbance (light scattering) at 320 nm was measured after 1 min stirring to allow the mixture to reach equilibrium. The increase in absorbance due to the scattered light was expressed as the percentage of relative maximum apparent absorbance (% rel. max. app. abs.) due to light scattering of the bound polyamine with DNA.

Cell Culture and Transfection Experiments

Two cell lines were used in the transfection experiment, a human primary skin fibroblast cells FEK4 (37) derived from a foreskin explant and a human cervix carcinoma, HeLa derivative and transformed cell line (HtTA). The HtTA cells being stably transfected with a tetracycline-controlled trans-activator (tTA) consisting of the tet repressor fused with the activating domain of virion protein 16 of the herpes simplex virus (HSV). Cells were cultured in Earle's minimal essential medium (EMEM) supplemented with foetal calf serum (FCS) 15% in the case of FEK4 and 10% in the case of HtTA cells, penicillin and streptomycin (50 IU/ml each), glutamine (2 mM), and sodium bicarbonate (0.2%).

For the transfection (gene delivery) and the resultant gene activity (transfection efficiency), FEK4 and HtTA cells were seeded at 1×10^5 cell/well in 6 well plates in 4 ml EMEM media with FCS for 24 h to reach a plate confluency of 50–60% on the day of transfection. The complex was prepared by mixing 2 μg of pEGFP with the cationic liposomes or lipopolyamine in Opti-MEM (serum free media, Gibco BRL) according to the charge ratio at room temperature for 30 min and then incubated with the cells for 4 h at 37°C in 5% CO_2. Then the cells were washed and cultured for further 44 h in growth medium at 37°C in 5% CO_2 before the assay.

Levels of enhanced green fluorescent protein (EGFP positive cells) in the transfected cells were detected and corrected for background fluorescence of the control cells using a fluorescence activated cell sorting (FACS) machine (Becton Dickinson FACS Vantage dual Laser Instrument, argon ion laser 488 nm). The transfection efficiency was calculated based on the percentage of the cells that expressed EGFP (positive cells) in the total number of cells.

Cytotoxicity (MTT) Assay of the Formed Lipoplexes

FEK4 and HtTA cells were seeded in 96 well plates at 8000 cell/well and incubated for 24 h at 37°C in 5% CO_2. N^4,N^9-Dioleoyl spermine complexed with pEGFP was added in the same way as the transfection protocol. After incubation for 44 h, the media was replaced with 90 μl of fresh media and

10 µl of sterile filtered MTT solution (Sigma-Aldrich, UK) (5 mg/ml) to reach a final concentration of 0.5 mg/ml. Then the plates were incubated for a further 4 h at 37°C in an atmosphere of 5% v/v CO_2. After incubation, the media and the unreacted dye were aspirated and the formed blue formazan crystals were dissolved in 200 µl/well of dimethyl sulfoxide (DMSO). The produced color was measured using a plate-reader (VERSAmax) at wavelength 570 nm. The % viability related to control wells containing cells without DNA and/or polymer and is calculated by (test absorbance/control absorbance) × 100 (38). The same protocol was applied in case of the commercially available reagents Lipofectin and Lipofectamine.

RESULTS

Synthesis of N^4, N^9-Dioleoyl Spermine

The synthesized N^4,N^9-dioleoyl spermine (Fig. 3) was homogenous on silica gel thin-layer chromatography and was fully characterized by ^{1}H-NMR (at 400 MHz) and ^{13}C-NMR and high-resolution accurate mass spectroscopy.

Ethidium Bromide Fluorescence Quenching Assay

To study the ability of N^4,N^9-dioleoyl spermine to condense calf thymus DNA and pEGFP as well as compared this effect with the effect of different known polycations spermine, PLL (39) and PEI (40) (Fig. 2) to condense DNA. Figure 4 shows the ability of the studied polyamines to displace EthBr from DNA. The binding ability was in the order PEI 2K > N^4,N^9-dioleoyl spermine > PLL 9.6K > spermine according to the charge ratio. The charge ratio was calculated according to the ammonium/phosphate (+/−) ratio for: spermine, 202.35 g/mole with four nitrogen atoms that can be protonated; PLL 9.6K, one positive charge/lysine monomer; N^4,N^9-dioleoyl spermine, with two positive charges. In the case of PEI 2K the charge ratio was calculated as 25% of the amino groups in the polymer that can be protonated, assuming that 43.1 g/mol is the repeating unit of PEI that contains one nitrogen atom (41,42). It seems to be that there is no agreement in the literature for the calculation of the PEI/DNA ratio. Although it is calculated as PEI nitrogen/DNA phosphate (40) not as charge ratio, it is reported that every third atom of PEI polymer is a protonatable nitrogen (40), but one in five of the protonatable nitrogens in PEI are protonated at pH 7 (43). However, Wagner (11) reported that one of every seven nitrogens within PEI polymer is protonated at pH 7. The polycations (PEI 2K and PLL 9.6K) are known for their ability to condense DNA efficiently at a relatively low charge ratio compared to spermine. On the other hand, the results revealed that N^4,N^9-dioleoyl spermine is able to condense DNA at a lower charge ratio than PLL 9.6K and spermine (Fig. 4), and produces a 50% fluorescence decrease at charge ratio 0.52. Also, Fig. 5 shows DNA condensation ability of N^4,N^9-dioleoyl spermine in comparison with the commercially available, cationic lipid, liposomal formulations Lipofectin and Lipofectamine. All three cationic lipid formulations have the ability to condense completely DNA through the displacement of EthBr leading to fluorescence quenching. At lower charge ratios N^4,N^9-dioleoyl spermine has better ability to suppress the fluorescence than Lipofectin and Lipofectamine. On studying the effect of N^4,N^9-dioleoyl spermine on the type of DNA (calf thymus DNA, and plasmid pEGFP), it was found that there is no significant variation in the condensation ability of the studied lipopolyamine on the type of DNA.

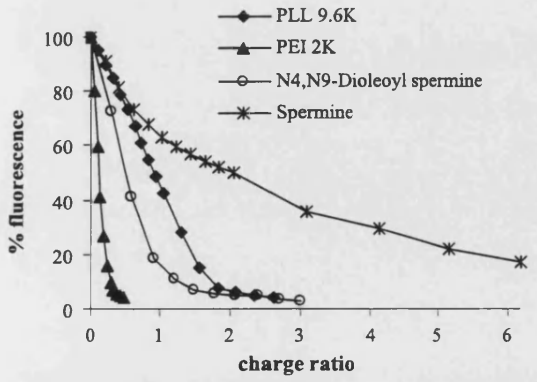

Fig. 4. Plot of EthBr displacement assay of calf thymus DNA complexed with PLL 9.6K, PEI 2K, N^4,N^9-dioleoyl spermine, and spermine.

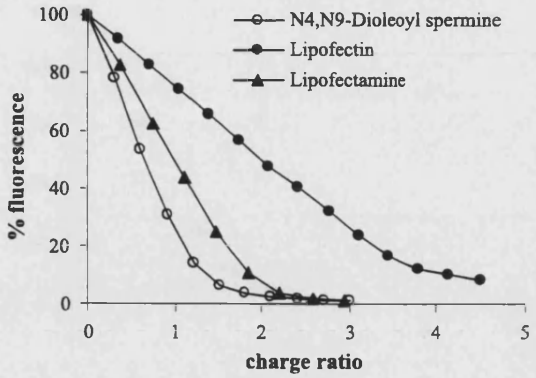

Fig. 5. Plot of EthBr displacement assay of calf thymus DNA complexed with N^4,N^9-dioleoyl spermine, Lipofectin, and Lipofectamine.

Light Scattering Assay

This experiment has been carried out to investigate the condensation of DNA by polyamines and the formation of particles (44). The apparent UV absorbance at 320 nm (where there is no DNA absorbance above 300 nm) was measured (28,45,46) showing light scattering. The results from Fig. 6 indicated the formation of particles upon interaction of spermine, PLL 9.6K and PEI 2K. In addition, Fig. 7 shows that the light scattering due to particle formation increases with the increase in the displaced EthBr and reaches the maximum at approximately the same charge ratio at which there is a maximum EthBr displacement, although the concentration of DNA used in light scattering experiments is ten times the concentration used in fluorescence quenching experiments which is related to the lack of sensitivity of light scattering experiment in comparison with fluorescence assay. Also, from light scattering results, there is a decrease in the % relative maximal apparent absorbance (% rel. max. app. abs.) after reaching the maximum absor-

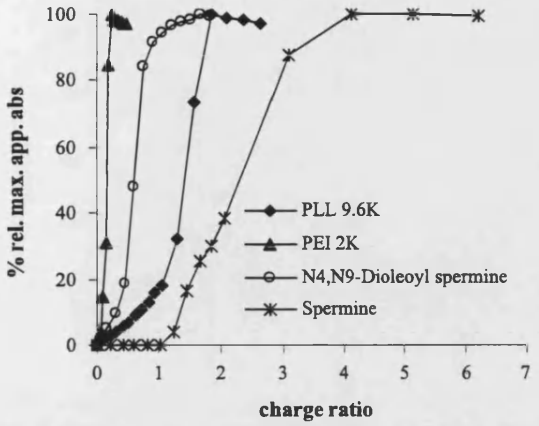

Fig. 6. Light scattering assay (% relative maximum apparent absorbance at λ = 320 nm) of calf thymus DNA complexed with PLL 9.6K, PEI 2K, N^4,N^9-dioleoyl spermine and spermine.

bance, which could be attributed to the formation of polyamine-DNA aggregates as reported by Gosule and Schellman (47).

Transfection Experiments

The transfection efficiency of pEGFP into FEK4 primary cell line and the cancer HtTA cells was studied using N^4,N^9-dioleoyl spermine and the commercially available reagents (Lipofectin and Lipofectamine). The transfection results of pEGFP into FEK4 indicated higher transfection ability of N^4,N^9-dioleoyl spermine (75%) and Lipofectamine (66%) formulations over Lipofectin (18%); there is no significant difference in the transfection activity between N^4,N^9-dioleoyl spermine and Lipofectamine (Fig. 8). On the other hand, both N^4,N^9-dioleoyl spermine and Lipofectamine formulations show a similar transfection activity in HtTA cells (about 70%), higher than Lipofectin (58%). N^4,N^9-Dioleoyl spermine transfects the cells best at charge ratio (+/−) 2.5 (5.54 μg/ml), Lipofectin at charge ratio 0.6 (5.0 μg/ml), while Lipofectamine transfect both cell lines at charge ratio 3.7 (10.0 μg/ml).

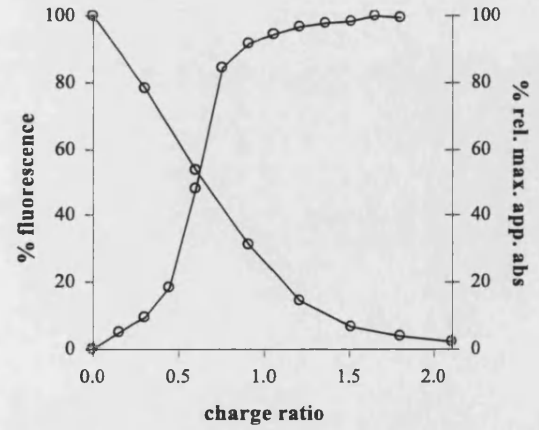

Fig. 7. Comparison of EthBr displacement and light scattering assays of calf thymus DNA with N^4,N^9-dioleoyl spermine.

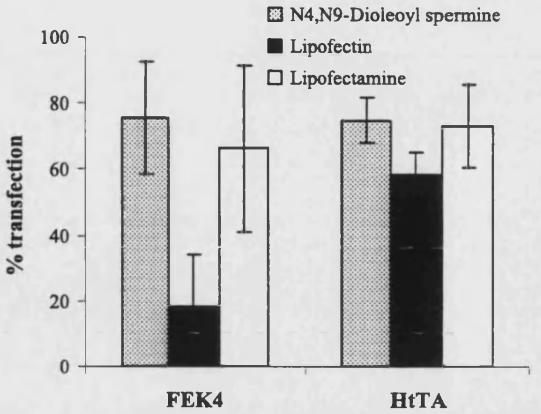

Fig. 8. Lipofection of FEK4 and HtTA cells transfected with pEGFP complexed with N^4,N^9-dioleoyl spermine. Lipofectin or Lipofectamine (at their respective N/P ratios for best transfection). The data represent 3 different experiments (3 replicates each) and the error bars represent the standard deviation.

In Vitro Cytotoxicity

The cytotoxicity of N^4,N^9-dioleoyl spermine was studied in FEK4 and HtTA cells using MTT assay (48) (Fig. 9). The IC_{50} (the concentration at which cell growth is inhibited by 50%) (42) values for the free polycation in FEK4 and HtTA

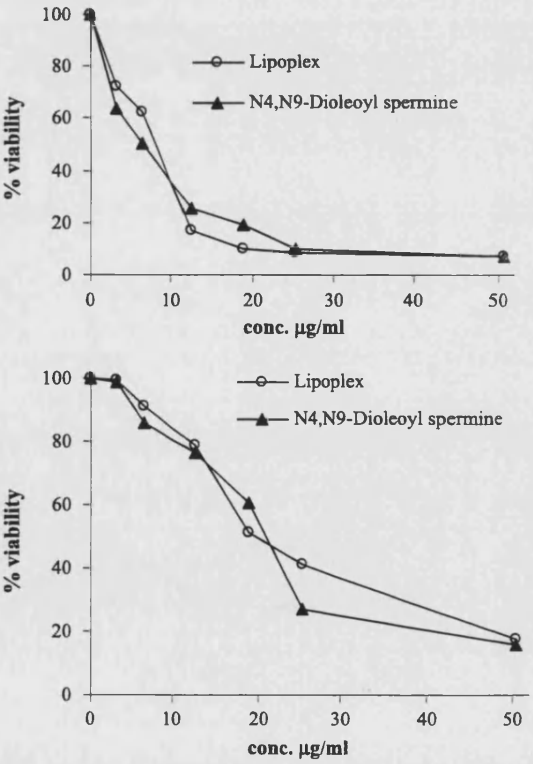

Fig. 9. Viability of HtTA cells (above) and FEK4 primary skin cells (below) after application of different concentrations of N^4,N^9-dioleoyl spermine either free or complexed with pEGFP.

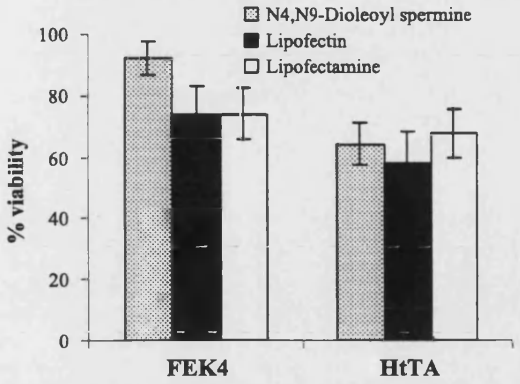

Fig. 10. Cytotoxicity effect of pEGFP (2 μg/ml) complexed with either N^4,N^9-dioleoyl spermine (5.54 μg/ml), Lipofectin (5 μg/ml) or Lipofectamine (10 μg/ml) in FEK4 and HtTA cells.

cells were 20.47 and 6.31 μg/ml respectively, and for the lipoplex were 19.82 and 8.35 μg/ml respectively. The results indicate that there is no significant difference in the toxic effect (IC_{50}) of the free polycation over the lipoplex in the case of HtTA cells and FEK4 cells for the studied lipopolyamines. N^4,N^9-Dioleoyl spermine show a significant difference in the % viability comparing to either Lipofectin and Lipofectamine in the primary skin cell line FEK4, but there is no difference in the case of the cancer cell line HtTA (Fig. 10). These results also revealed that N^4,N^9-dioleoyl spermine toxicity is higher (lower concentrations) in the case of HtTA cells more than with the primary cell line FEK4 which could be attributed to the ease of transfection of immortalized cancer cell lines over primary cell lines.

DISCUSSION

Non-viral delivery systems can be defined to include the use of plasmid DNA alone (so-called naked DNA) (49,50) as well as DNA complexed to synthetic carriers such as cationic lipids (51–54) or polymers (55). The use of an efficient carrier for nucleic acid delivery is considered to be a determinant factor for the successful application of gene therapy (56). This carrier is responsible for the complex process of gene delivery to the nucleus (57).

Ethidium bromide (EthBr) (2,7-diamino-10-ethyl-9-phenylphenanthridinium bromide, Fig. 11) is a cationic dye that displays a marked increase in the fluorescence upon binding with DNA and RNA through the intercalation between the EthBr phenanthridinium-moiety and adjacent base-pairs of DNA sequences (36,58). Within the prerequisites for delivery of DNA across intact cytoplasmic membrane are condensation and masking the negative charge of the phosphate backbone. Condensation of DNA occurs when about 90% of the charge on DNA is neutralized (16,47).

The ability of the cationic lipid N^4,N^9-dioleoyl spermine to compact DNA more efficiently than both spermine and the powerful condensing agent PLL a cationic polymer (9), (Fig. 4) shows the importance of the lipid moiety we have bound to the cationic polyamine in order to achieve improvements in its ability to condense DNA, cellular entry, and lowering the toxicity of the polyamine conjugate (11,59,60).

The formation of N^4,N^9-dioleoyl spermine-DNA lipoplex at lower charge ratio decreases the toxicity of the DNA delivering lipopolyamine. As the mammalian cell membrane is a semipermeable membrane formed of phospholipids bilayer that allows the transport of macromolecules by endocytosis, neutralization of the negative charges on the DNA by polycations will improve the delivery of DNA through the cell membrane because of the presence of negative charges on both DNA and cell membrane. Also, the positively charged lipid complex will mediate transfection by fusion with cell membranes (60,61). It was found that both the number of positive charges and their distribution on the surface of the molecule have profound effects on DNA condensation (28,62,63).

In addition, in a study on the transfection activity of cholesterol carbamate cationic lipids (28), Blagbrough and co-workers reported that the carbamate with a spermine polyamine moiety has the highest transfection activity with its ability to condense DNA efficiently. Our findings are in agreement with the literature, the cationic liposomal formulation Lipofectin, with its cationic moiety DOTMA containing one positively charged quaternary ammonium group, has lower ability to displace the EthBr from DNA than Lipofectamine formulation that contains DOSPA (Fig. 2) with its four positively charged nitrogens (15) and N^4,N^9-dioleoyl spermine with its two positively charged nitrogens. The higher ability of N^4,N^9-dioleoyl spermine (two positive charges) over Lipofectamine (four positive charges) in DNA condensation, though N^4,N^9-dioleoyl spermine has a lower number of positive charges/molecule, may be attributed to the distribution of the positive charges on the molecule allowing a higher affinity of the vector for DNA and leading to the efficient displacement of EthBr. Another variable is the liposomal formulation of Lipofectamine compared to the non-liposomal formulation of N^4,N^9-dioleoyl spermine.

The helper lipid DOPE which is the second component of the cationic liposomes is used to increase the transfection activity of the cationic liposome through its ability to destabilize lipid bilayers leading to endosomal destabilization with subsequent increase in the total cellular uptake of the delivered DNA (23,64). Lipospermines (Fig. 2) with their cationic headgroup sometimes form micelles (in the absence of DNA) rather than the bilayer produced by the small cationic quaternary ammonium headgroup of DOTMA (Lipofectin formulation) (43,65). N^4,N^9-Dioleoyl spermine combines in its structure the two oleoyl chains that have the characteristics of the fusogenic lipid DOPE and the cationic polyamine spermine (Fig. 2). Thus, DNA is condensed by the

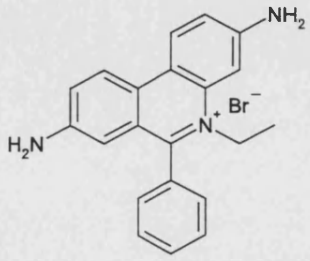

Fig. 11. Structure of ethidium bromide.

two primary amines and coated by the dioleoyl lipophilic moiety. The DNA condensation results of N^4,N^9-dioleoyl spermine (Fig. 5) show a higher efficiency than either liposomal formulation. The transfection results reveal higher transfection ability (levels of expression) of N^4,N^9-dioleoyl spermine compared to Lipofectin (Fig. 8) in FEK4 and HtTA cells. These results indicate the roles of both number and distribution of positive charges along the polyamine backbone on the ability of the compound to condense DNA. On the other hand, there is no significant difference in the transfection activity between N^4,N^9-dioleoyl spermine and Lipofectamine in both FEK4 and HtTA cell lines, which indicates the importance of lipid coating over the DNA molecule on both the condensation and cellular delivery of DNA in case of the liposomal and non liposomal formulations (65). N^4,N^9-Dioleoyl spermine achieved high levels of transfection in both a cancer cell line and a primary skin cell line (73%), which indicates the ability of this vector to deliver DNA. Cell viability results revealed improved FEK4 viability more than the cancer cells HtTA (Fig. 9). Also, N^4,N^9-dioleoyl spermine showed a significant improvement in primary FEK4 cell viability over the liposomal formulatios (Fig. 10), but no significant difference in HtTA cells. Previous studies on Lipofectin and Lipofectamine on different cell lines showed the cytotoxic effects of these liposomal formulations (66–70). In conclusion, a lipopolyamine vector has been developed for DNA delivery. This new non-liposomal formulation has the ability to transfect primary skin cells more efficiently than the commercially available liposomal Lipofectin formulation.

ACKNOWLEDGMENTS

We acknowledge the financial support of the Egyptian Government studentship to O.A.A. A. and Universities U.K. for an ORS award to N. A. We are grateful to Prof R.M. Tyrrell (University of Bath) for FEK4 and HtTA cell lines.

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Pharmaceutical Research, Vol. 23, No. 1, January 2006 (© 2006)
DOI: 10.1007/s11095-005-8717-3

Feature Article

Varying the Unsaturation in N^4,N^9-Dioctadecanoyl Spermines: Nonviral Lipopolyamine Vectors for More Efficient Plasmid DNA Formulation

Osama A. A. Ahmed,[1] Charareh Pourzand,[1] and Ian S. Blagbrough[1,2]

Received August 10, 2005; accepted September 23, 2005

Purpose. The aim of the study is to analyze the effect of varying the degree of unsaturation in synthesized N^4,N^9-dioctadecanoyl spermines on DNA condensation and then to compare their transfection efficiency in cell culture.
Methods. The N^4,N^9-di-C18 lipopolyamines—saturated (stearoyl), C9-*cis*- (oleoyl), and C9,12-di-*cis*-(linoleoyl)—were synthesized from the naturally occurring polyamine spermine. The ability of these novel compounds to condense DNA and form nanoparticles was studied using ethidium bromide fluorescence quenching and nanoparticle characterization techniques. Transfection efficiency was studied in several primary skin cells (FEK4, FCP4, FCP5, FCP7, and FCP8) and in an immortalized cancer cell line (HtTA) and was compared with the commercially available nonliposomal transfection formulation Transfectam® (dioctadecylamidoglycyl spermine), which also contains two saturated C18 lipid chains.
Results. N^4,N^9-Dilinoleoyl spermine (C18, di-*cis*-9,12) is efficient at circular plasmid DNA (pEGFP) condensation and gives the most effective transfection in a series of primary skin cells and cancer cell lines at low charge ratios of 5.5 (± ammonium/phosphate).
Conclusions. The dienoic fatty acyl spermine conjugate N^4,N^9-dilinoleoyl spermine efficiently condenses DNA and achieves the highest transfection levels among the studied lipopolyamines in cultured cells.

KEY WORDS: FCP; FEK4; gene delivery; HtTA; lipopolyamine; N^4,N^9-dilinoleoyl spermine; N^4,N^9-dioleoyl spermine; N^4,N^9-distearoyl spermine; primary skin cells; transfection.

INTRODUCTION

As viral gene therapy continues to suffer significant problems with mammalian toxicity (1–3), the possibility of reaching the goal of intracellular protein levels at therapeutic concentrations moves even more toward utilizing nonviral gene therapy (NVGT). Within this broad term, we are concentrating on lipopolyamines composed of a lipophilic steroid attached either to a polyamine chain (4–8) or to a single or double long-carbon chain covalently bound to a polyamine, e.g., spermine (N^1,N^{12}-diamino-4,9-diazadodecane) (9–11). Other research groups are investing in a variety of alternative approaches classified under the NVGT umbrella, including the following: naked DNA (12), gene gun (bound to gold particles) (13,14), electroporation (15), polycation-mediated DNA delivery, and the use of a wide variety of cationic lipids (lipoplexes) (16), e.g., bolaamphiphiles (17), and cationic polymers (polyplexes) (18–20) [for reviews, see (21–25)]. For gene therapy to realize its potential and become an efficient medicine for the treatment of diseases such as cancer, cystic fibrosis, inflammation, or for vaccination, key obstacles must be overcome. The essential requirements for gene delivery are the transport of DNA through the cell membrane and ultimately to the nucleus. The design of an efficient formula for the delivery of genetic material requires a detailed understanding of the barriers that hinder this process. Thus, efficient formulations, lipoplexes (16,17), and polyplexes (18–20) must be able to deliver safely the required DNA across the various cellular barriers to the nucleus. Barriers to DNA delivery also include complex formation between the DNA and the lipopolyamine leading to DNA nanoparticle formation by electrostatic charge neutralization (to about 90% of the total negative charge) and overall packing as condensed DNA nanoparticles (26). So, a key first step in this method of gene formulation is masking the negative charges of the phosphate backbone. This titration with a lipopolyamine causes alleviation of charge repulsion between remote phosphates along the DNA helix leading to collapse into a more compact structure that facilitates cell entry.

For NVGT, except for naked (free, uncomplexed) DNA being trapped inside cells by direct association with the

[1] Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, UK.
[2] To whom correspondence should be addressed. (e-mail: prsisb@bath.ac.uk)

ABBREVIATIONS: DOGS, dioctadecylamidoglycyl spermine; DOPC, dioleoylphosphatidyl choline; DOPE, dioleoylphosphatidyl ethanolamine; EGFP, enhanced green fluorescent protein; EMEM, Earle's Minimal Essential Medium; EthBr, ethidium bromide; FCS, fetal calf serum; HRMS, high-resolution mass spectroscopy; NVGT, nonviral gene therapy; PE, phosphatidyl ethanolamine; PLL, poly-L-lysine.

chromatin during mitosis, the (prodrug) DNA must be formulated. Small molecule synthetic cationic lipids are one of the major gene carriers for NVGT, often classified as liposomal and nonliposomal nonviral delivery vectors. They condense DNA into nanoparticles that are readily endocytosed by cultured cells and facilitate endosomal escape leading to efficient delivery to the nucleus presumably crossing through the nuclear pore complex. After nuclear entry, the payload DNA should ultimately be able to give the desired protein through transcription and translation.

The first cellular barrier for the delivery of the DNA nanoparticles is the eukaryotic cell membrane, which is composed mainly of phospholipids (50–90% of total lipid content; most phospholipids are derivatives of diacyl-glycerol-3-phosphate), sterols (5–25%), and glycol lipids (usually less than 5%). The diacyl-glycerol chains (C14–C24) are derived from linear fatty acids with varying degrees of unsaturation (27). In NVGT, cationic lipids, which interact with the DNA payload, also mediate cell-membrane transport, typically through adsorptive endocytosis or mediated by cations (28–30), both routes leading to internalization of the DNA complex nanoparticles. Such endocytosis is via the clathrin-coated pits (250–300 nm) (31), followed by fusion of the early endosome and sorting to the late endosomal compartment, hence avoiding degradation in the lysosome. One of the important factors improving the release of free DNA or the lipoplex into the cytoplasm is the influence of the cationic lipid chain. Xu and Szoka (32) have reported that unsaturated hydrocarbon chains increase the transfection efficiency of the lipoplex by decreasing the rigidity of the bilayer and favoring a higher intermembrane transfer rate and lipid mixing, compared with their saturated counterparts. However, Roosjen *et al.* (33) recently reported that, in certain cases, saturated fatty chains afforded better results than unsaturated chains. Herein, we investigate the effects on DNA formulation of a circular plasmid with variation in the degree of unsaturation in the two C18 fatty chains in our lipospermines, the saturated N^4,N^9-distearoyl spermine, the alkenoic N^4,N^9-dioleoyl spermine, and the dienoic fatty acyl spermine conjugate N^4,N^9-dilinoleoyl spermine. We report the synthesis and characterization of the nanoparticles, summarize transfection results with the synthesized lipospermine formulations in a panel of both primary and cancer cell lines, and compare these results with those obtained with the commercially available nonliposomal lipospermine Transfectam® [dioctadecylamidoglycyl spermine (DOGS)] formulation (34,35).

MATERIALS AND METHODS

Materials

Chemicals, including spermine, acyl chlorides (linoleoyl, oleoyl, and stearoyl chlorides), solvents, buffers, and DNA were routinely purchased from Sigma-Aldrich, Dorset, UK, except where indicated. Transfectam® was from Promega (Southampton, UK), and cell culture materials were from Life Technologies (Paisley, Scotland).

Preparation of Plasmid DNA

We have chosen to deliver a 4.7-kbp plasmid encoding for enhanced green fluorescent protein (pEGFP), with a molecular weight of about 3.1 MDa (given an average of 330 Da per nucleotide, 660 Da per base pair (36), carrying 9400 negative charges). DNA plasmid pEGFP, purchased from Clontech (Basingstoke, UK), was transformed into *Escherichia coli* JM 109 bacterial strain (Promega) and was purified by a Qiagen Maxi kit (Qiagen, Chatsworth, CA, USA). DNA yields and purity were determined spectroscopically (OD_{260}/OD_{280} = 1.80–1.90 OD, optical density) and by agarose gel (1%) analysis.

Synthesis of Lipospermines N^4,N^9-Dilinoleoyl Spermine, N^4,N^9-Dioleoyl Spermine, and N^4,N^9-Distearoyl Spermine

Spermine was used as the starting material for the synthetic process, outlined in Fig. 1. The tetra-amine was protected on both the primary amino functional groups with ethyl trifluoroacetate (2.2 eq.) in methanol, and the reaction mixture was stirred for 18 h at 20°C. The solvent was evaporated to dryness *in vacuo* to form N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane. Fatty acyl chloride (linoleoyl, oleoyl, and stearoyl; 2.2 eq) and triethylamine (2.5 eq) were added to the diprotected spermine solution in CH_2Cl_2 and methanol (1:1). The solution was stirred for 72 h at 20°C and then evaporated to dryness *in vacuo*. The residue was dissolved in CH_2Cl_2, and the solution was filtered and evaporated to dryness *in vacuo* to form N^4,N^9-[dilinoleoyl, dioleoyl (37), or distearoyl]-N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane. For the removal of the ditrifluoroacetyl protecting groups, the tetraamide was dissolved in methanol, and the pH of the solution was increased by saturating with ammonia gas, then it was left

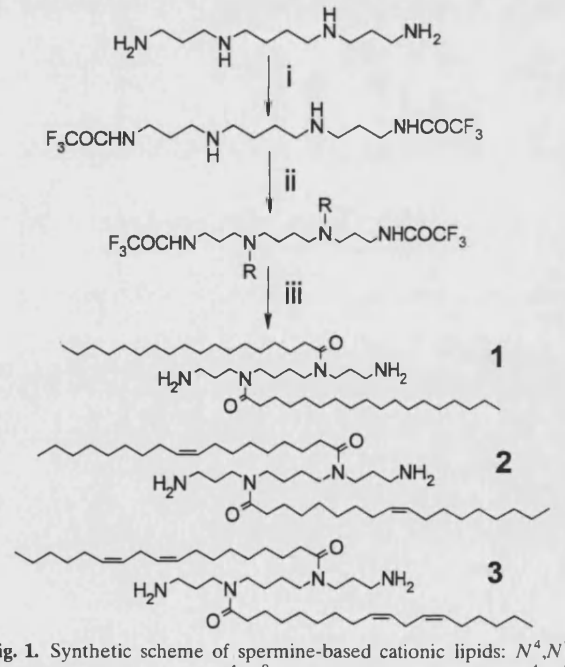


Fig. 1. Synthetic scheme of spermine-based cationic lipids: N^4,N^9-distearoyl spermine **1**, N^4,N^9-dioleoyl spermine **2**, and N^4,N^9-dilinoleoyl spermine **3**. Reagents: (i) ethyl trifluoroacetate, methanol, 18 h at 20°C; (ii) fatty acyl chloride, R = (stearoyl **1**, oleoyl **2**, and linoleoyl **3**), triethylamine, CH_2Cl_2, and methanol (1:1), 72 h at 20°C; (iii) methanol saturated with ammonia gas, 18 h at 20°C.

for 18 h at 20°C and then evaporated to dryness *in vacuo* to give a residue that was purified over silica gel (CH_2Cl_2–MeOH 5:3, v/v, then CH_2Cl_2–MeOH–conc. aq. NH_3 25:10:1, v/v/v) to afford the three desired lipopolyamine conjugates as their free bases. All three synthesized lipopolyamines were homogenous on silica gel thin-layer chromatography (CH_2Cl_2–MeOH–conc. aq. NH_3 25:10:1, v/v/v) and were characterized by ^{1}H-nuclear magnetic resonance (NMR; at 400 MHz) and ^{13}C-NMR spectroscopy (assignments follow from correlation spectroscopy) and by high-resolution mass spectroscopy (HRMS):

N^4,N^9-distearoyl spermine **1**, R_f = 0.41, NMR *inter alia* (C^2HCl_3) δ ^{1}H 0.91 ppm (t, 2 × CH_3), 1.25–1.75 ppm (br m, many × CH_2), 2.40–2.58 ppm (br s, 2 × NH_2), and 3.24–3.48 ppm (br m, many × NCH_2), δ ^{13}C 173 ppm (2 × CON), $C_{46}H_{95}N_4O_2$ found (*m/z* [M + H]$^+$) 735.7458, requires 735.7455 (Δ ppm −0.4), $C_{45}{}^{13}CH_{95}N_4O_2$ found 736.7500, requires 736.7489 (Δ ppm −1.5).

N^4,N^9-dioleoyl spermine **2**, R_f = 0.44, NMR *inter alia* (C^2HCl_3) δ ^{1}H 0.91 ppm (t, 2 × CH_3), 1.24–1.70 ppm (br m, many × CH_2), 2.03 ppm (app q, 4 × allylic CH_2), 4.55–5.10 ppm (br s, 2 × NH_2), and 5.32–5.41 ppm (m, 2 × CHCH), δ ^{13}C 130 ppm (2 × CHCH) and 173 ppm (2 × CON), $C_{46}H_{91}N_4O_2$ found (*m/z* [M + H]$^+$) 731.7115, requires 731.7142 (Δ ppm 3.7), $C_{45}{}^{13}CH_{91}N_4O_2$ found 732.7145, requires 732.7176 (Δ ppm 4.1).

N^4,N^9-dilinoleoyl spermine **3**, R_f = 0.49, NMR *inter alia* (C^2HCl_3) δ ^{1}H 0.89 ppm (t, 2 × CH_3), 1.25–2.00 ppm (br m, many × CH_2), 2.05 ppm (app q, 4 × allylic CH_2), 2.77 ppm (app t, 2 × doubly allylic CH_2), 5.29–5.42 ppm (m, 4 × CHCH), and 5.85–6.20 ppm (br s, 2 × NH_2), δ ^{13}C 128 and 130 ppm (4 × CHCH) and 174 ppm (2 × CON), $C_{46}H_{87}N_4O_2$ found (*m/z* [M + H]$^+$) 727.6846, requires 727.6829 (Δ ppm −2.3), $C_{45}{}^{13}CH_{87}N_4O_2$ found 728.6857, requires 728.6863 (Δ ppm 0.7).

DNA Condensation

DNA condensation was monitored using an ethidium bromide (EthBr) fluorescence quenching assay as described in detail earlier (11). Briefly, DNA (6 μg) was diluted to 3 ml with buffer [20 mM NaCl, 2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 μM ethylenediaminetetraacetic acid (EDTA), pH 7.4] in a glass cuvette stirred with a microflea. EthBr solution (3 μl, 0.5 mg/ml) was added to the stirring solution and was allowed to equilibrate for 10 min. Separately, lipopolyamine aliquots [5 μl, according to the ammonium/phosphate (±) charge ratio required] were then added to the stirring solution, and the fluorescence was measured after 1-min equilibration using a Perkin-Elmer LS 50B luminescent spectrometer (λ_{excit} = 260 nm and λ_{emiss} = 600 nm with slit width 5 nm) while stirring using an electronic stirrer (Rank Bros. Ltd., Bottisham, Cambridge, England). The fluorescence was expressed as the percentage of the maximum fluorescence when EthBr was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free EthBr in solution.

Also, each sample of plasmid DNA (0.5 μg), either free or complexed with the lipopolyamine, was analyzed by gel electrophoresis for about 60 min under 75 V/cm, through an agarose gel (1%) containing EthBr (1 μg/ml) in Tris–acetate–EDTA 1× (40 mM Tris–acetate and 1 mM EDTA) buffer. The (unbound) free DNA in the agarose gel was visualized under UV using GeneGenius (Syngene, Cambridge, UK).

Lipoplex Particle Size and ζ-Potential Measurements

The average particle size for the lipoplexes formed (at their optimum charge ratio of transfection), after mixing with a vortex mixer, was determined using a Malvern Zetasizer (Nano S, Malvern Instruments, Malvern, UK), and ζ-potential measurements were determined using a Malvern Zetasizer (Nano ZS, Malvern Instruments). All measurements were carried out on lipoplexes with 5 μg/ml plasmid DNA in HEPES buffer at pH 7.4 and 20°C.

Cell Culture and Transfection Experiments

Six cell lines were used in the transfection experiment; FEK4 (38), FCP4, FCP5, FCP7, and FCP8 cells are human primary fibroblasts derived from newborn foreskin explants (39). HtTA cells are a human cervical carcinoma, HeLa-derived and transformed cell line. Cells were cultured in Earle's Minimal Essential Medium (EMEM) supplemented with fetal calf serum (FCS), 15% in the case of FEK4 and FCP cells and 10% in the case of HtTA cells, penicillin and streptomycin (50 IU/ml each), glutamine (2 mM), and sodium bicarbonate (0.2%). Primary cells were passaged once a week and used between passages 7 and 15.

For the transfection (gene delivery) and the resultant gene activity (transfection efficiency), cells were seeded in 6-well plates at a density of 1 × 10^5 cells/well (in 4 ml EMEM with FCS) for 24 h to reach a 50–60% confluency on the day of transfection. The complex was prepared by mixing pEGFP (2 μg/ml/well) with each lipopolyamine in Opti-MEM (serum-free media, Gibco BRL, Berlin, Germany) according to the charge ratio at 20°C for 30 min and then incubated with the cells for 4 h at 37°C in 5% CO_2. Then the cells were washed and cultured for a further 44 h in growth medium at 37°C in 5% CO_2.

Levels of EGFP in the transfected cells were detected and corrected for background fluorescence of the control cells using a fluorescence-activated cell sorting (FACS) machine (Becton Dickinson FACS Vantage dual Laser Instrument, argon ion laser 488 nm). The transfection efficiency was calculated based on the percentage of the cells that expressed EGFP (positive cells) in the total number of cells.

In Vitro Cytotoxicity

Briefly, FEK4, FCPs, and HtTA cells were seeded in 96-well plates at 8000 cells/well and incubated for 24 h at 37°C in 5% CO_2. Lipoplexes with pEGFP were added at the same concentration as in the transfection protocol (2 μg/ml). Cytotoxicity was evaluated after incubation for 44 h; sterile-filtered 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (10 μl, 5 mg/ml; Sigma-Aldrich, UK) was added to reach a final MTT concentration of 0.5 mg/ml. The color produced was measured using a plate

reader (VERSAmax™) at λ = 570 nm. The percent viability related to control wells containing untreated cells (without naked DNA and without lipopolyamine–DNA lipoplex) is calculated by [OD (measured)/OD (untreated cells)] × 100 (40). The same protocol was followed for the commercially available reagent Transfectam® (34,35).

RESULTS

Synthesis of Lipospermines

The tetra-amine spermine was used as the starting material for the synthesis of the three desired lipopolyamines: N^4,N^9-distearoyl spermine **1**, N^4,N^9-dioleoyl spermine **2**, and N^4,N^9-dilinoleoyl spermine **3** (Fig. 1). The tetra-amine was protected on both the primary amino functional groups with ethyl trifluoroacetate (2.2 eq.) in methanol. Each corresponding fatty acyl chloride (stearoyl, oleoyl, or linoleoyl) was used as the acylating agent together with triethylamine in CH_2Cl_2 and methanol (1:1). Deprotection in methanol saturated with ammonia gas and flash column chromatography afforded the three target lipospermines, homogenous on silica gel thin-layer chromatography, N^4,N^9-dilinoleoyl spermine R_f = 0.49, N^4, N^9-dioleoyl spermine R_f = 0.44, and N^4,N^9-distearoyl spermine R_f = 0.41 (CH_2Cl_2–MeOH–conc. aq. NH_3 25:10:1, v/v/v), and fully characterized by ^{1}H-NMR (at 400 MHz) and ^{13}C-NMR spectroscopy and by HRMS.

DNA Condensation

In Fig. 2, we show the DNA condensation ability of the synthesized lipopolyamine formulations in comparison with the commercially available Transfectam® in an EthBr fluorescence quenching assay. Three cationic lipid formulations (N^4,N^9-dilinoleoyl spermine, N^4,N^9-dioleoyl spermine, and Transfectam®) show similar DNA condensing ability. They are able to condense DNA completely [defined as more than 90% EthBr fluorescence quenching (26,41)] at N/P charge ratio 2, whereas N^4,N^9-distearoyl spermine is only able to displace around 80% of EthBr at the same charge ratio.

The binding of our three synthetic lipopolyamines with polyanionic DNA was also studied by analysis of the electrophoretic mobility of the circular plasmid DNA within an agarose gel (1%). All four spermine conjugates (including Transfectam®) were able to condense pEGFP DNA efficiently (as a result of the interactions between the lipopolyamine ammonium ions and the DNA phosphate charges) at their optimized respective charge ratios (N/P) of transfection (see Table I). All these conjugates completely inhibited the electrophoretic mobility of circular plasmid DNA from lipoplexes at these optimized N/P charge ratios.

Table I. Particle Size and Polydispersity of pEGFP Complexes with the Studied Lipopolyamines

Lipospermine	Charge ratio (N/P)	Lipoplex diameter (nm)	Polydispersity
N^4,N^9-Distearoyl spermine	15.0	217 (21)	0.46
N^4,N^9-Dioleoyl spermine	2.5	366 (73)	0.32
N^4,N^9-Dilinoleoyl spermine	5.5	71 (2.5)	0.19
Transfectam®	5.9	62 (3.2)	0.22

Each lipoplex diameter value represents mean ± SD.

Lipoplexes Particle Size and ζ-Potential Measurements

The particle size and ζ-potential characterization measurements were carried out on the lipoplexes at their optimum N/P charge ratio of transfection (see Table I). Particle size characterization by dynamic light scattering showed that the average particle size of N^4,N^9-dilinoleoyl spermine was considerably smaller (71 nm) than those of N^4,N^9-distearoyl spermine and N^4,N^9-dioleoyl spermine (Table I). The surface charge, as determined by ζ-potential measurements, was +43 and +32 mV for N^4,N^9-dilinoleoyl spermine (at N/P charge ratio of 5.5) and N^4,N^9-dioleoyl spermine (at N/P charge ratio of 2.5) lipoplexes, respectively (Fig. 3). Transfectam® lipoplex shows a ζ-potential of +20 mV (at N/P charge ratio of 6) in phosphate-buffered saline solution (Remy and Behr, personal communication).

ζ-Potential is an important parameter helping to predict the stability of the formulation as well as the ability of the positively charged particles to interact with cell membranes

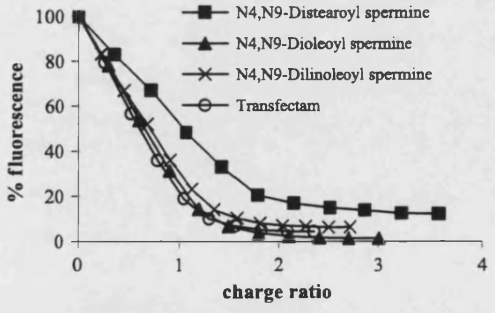


Fig. 2. Plot of EthBr displacement assay of calf thymus DNA complexed with N^4,N^9-dilinoleoyl spermine, N^4,N^9-dioleoyl spermine, N^4,N^9-distearoyl spermine, and Transfectam®.

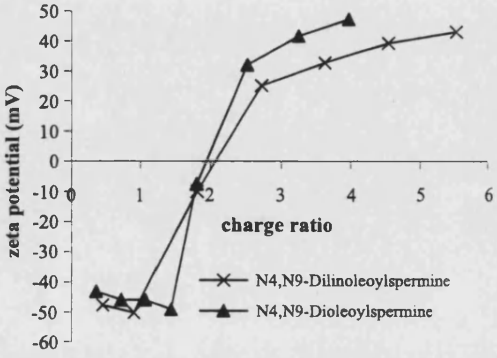


Fig. 3. ζ-Potential of N^4,N^9-dilinoleoyl spermine and N^4,N^9-dioleoyl spermine complexed with plasmid encoding for enhanced green fluorescent protein (pEGFP) at different N/P charge ratios.

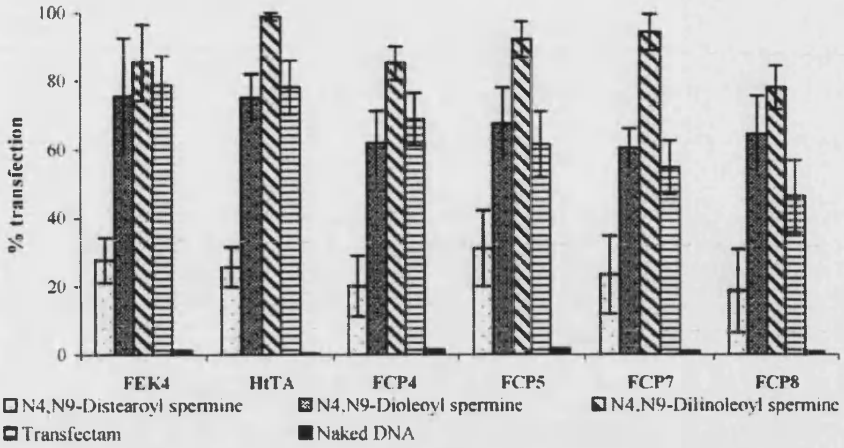


Fig. 4. Lipofection of the primary skin cell lines (FEK4, FCP4, FCP5, FCP7, and FCP8) and the cancer cell line HtTA transfected with pEGFP complexed with N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine, N^4,N^9-dilinoleoyl spermine, and Transfectam® (at their respective N/P ratios for best transfection). The data represent three different experiments (three replicates each), and the error bars represent SD.

(Remy and Behr, Personal Communication), (42). ζ-Potential depends on several factors, including pH, ionic charge, ion size, and concentration of ions in solution (43). The formed nanoparticles are considered to be stable when they have pronounced ζ-potential values, either positive or negative, but the tendency to aggregate is higher when the ζ-potential is close to zero. Charge neutrality ($\zeta = 0$) occurred at an N/P charge ratio of about 2 for both lipopolyamines N^4,N^9-dilinoleoyl spermine and N^4,N^9-dioleoyl spermine (comparable with our EthBr results; Fig. 2). Ciani *et al.* (43) found neutralization N/P charge ratios of 6 and 2 with intact liposomes. Similarly, also wor-king with liposomes, Lobo *et al.* (44) and Wiethoff *et al.* (45) reported that neutralization charge ratios of cationic lipid–DNA complexes occurred at N/P charge ratios of 3.5, 2.5, and 1.5.

Transfection Experiments

The transduction of EGFP into a series of primary skin cell lines (FEK4, FCP4, FCP5, FCP7, and FCP8) and a cancer cell line (HeLa-derived HtTA) was investigated. The optimum concentrations (and corresponding N/P charge ratios) for transfection were experimentally determined to be N^4,N^9-distearoyl spermine (33.4 μg/ml, N/P = 15.0), N^4,N^9-dioleoyl spermine (5.5 μg/ml, N/P = 2.5), N^4,N^9-dilinoleoyl spermine (12.1 μg/ml, N/P = 5.5), and Transfectam® (15.0 μg/ml, N/P = 5.9). The results indicate the improved transfection ability of N^4,N^9-dilinoleoyl spermine, greater than 85% in many of the studied primary cell lines (80% for FCP8 cells) and 99% in the case of the cancer cell line HtTA compared to the saturated N^4,N^9-distearoyl spermine (18–32%), the monounsaturated N^4,N^9-dioleoyl spermine (62–75%), and the commercially available Transfectam® (46–79%; Fig. 4). As a negative control, naked (uncomplexed circular) pEGFP DNA typically gave 1–2% transfection of these cell lines. Also, the shift in the EGFP positive cells compared to the untransfected cells in FACS analysis (Fig. 5) shows the high efficiency of N^4,N^9-dilinoleoyl spermine by transfecting both primary (e.g., FEK4) and cancer (HtTA) cell lines.

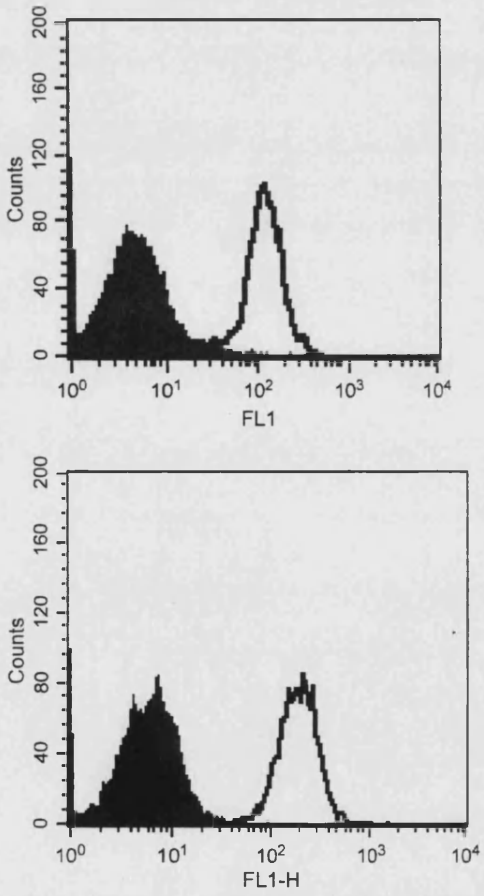


Fig. 5. Fluorescence-activated cell sorting analysis of FEK4 (above) and HtTA (below) cells after 48-h transfection of pEGFP complexed with N^4,N^9-dilinoleoyl spermine: ■, untransduced cells; □, EGFP positive cells.

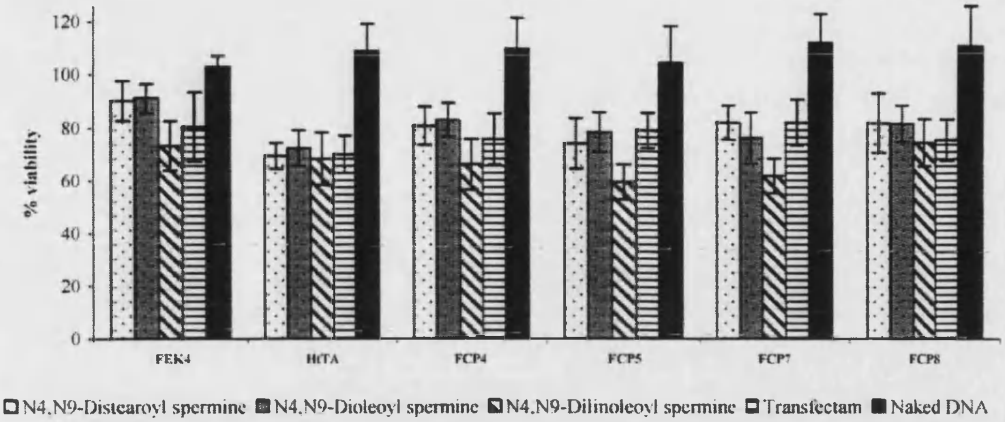


Fig. 6. Cytotoxicity effect of pEGFP (2 μg/ml) free (naked DNA) or complexed with N^4,N^9-distearoyl spermine (33.4 μg/ml), N^4,N^9-dioleoyl spermine (5.5 μg/ml), N^4,N^9-dilinoleoyl spermine (12.1 μg/ml), and Transfectam® (15.0 μg/ml) in the tested cells.

In Vitro Cytotoxicity

Cell viability [MTT assay (46)] results indicate that there is no significant difference in lipoplex toxicity in the case of HtTA and FCP8 primary skin cells for all four studied lipopolyamines (Fig. 6). Only small differences were observed across the other four cell lines, often with percentage cell viability about 80%. N^4,N^9-Dilinoleoyl spermine typically shows 70% cell viability in most of the studied cell lines, and such a value has recently been reported to be acceptable for a safe DNA delivery vector (47).

DISCUSSION

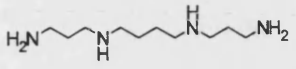
In this study, we have investigated the change in the degree of unsaturation of the di-C18 fatty chain formulation of lipospermine on DNA condensation and cellular delivery. The results from pEGFP condensation, investigated by EthBr fluorescence quenching assay, revealed that two of our synthetic lipopolyamines and the commercial formulation Transfectam® were able to condense DNA to less than 10% EthBr fluorescence, where DNA is defined as condensed (41); however, N^4,N^9-distearoyl spermine had not reduced (quenched) the EthBr fluorescence to 10% by N/P charge ratio 3.5 (Fig. 2). Particle size of the final gene formulation is also an important factor in improving gene delivery (48,49). Particle size results (Table I) showed larger particles with both N^4,N^9-dioleoyl spermine and N^4,N^9-distearoyl spermine formulations over those obtained with N^4,N^9-dilinoleoyl spermine and Transfectam®. These results are in concordance with the improvement in transfection achieved with N^4,N^9-dilinoleoyl spermine over our other two formulations. On the other hand, although Transfectam® has the smallest particle size (62 nm), the saturation of the di-C18 fatty chains could possibly contribute to the lower transfection efficiency of this spermine conjugate in comparison to our formulation results with N^4,N^9-dilinoleoyl spermine. Gao and Huang (51) reported that the addition of poly-L-lysine (PLL) or protamine to DC-Chol liposomes reduces the size of the complex as well as its heterogeneity in all ratios of lipid/DNA that improve transfection efficiency. Noguchi *et al.* (16), also working with protamine, reported that smaller-sized particles transfect cells efficiently. The incorporation of protamine as a nuclear localization signal to liposomes incorporating a cationic cholesterol derivative reduced the size of the formed particles from 0.4–1.8 μm, for the DNA–liposome complex, to 0.1–0.8 μm, for the DNA–protamine–liposome complex. On the relationship between particle size and transfection efficiency, there are no definite limits to the nanoparticle size that are suitable for transfection (51). Nanoparticles have relatively higher intracellular uptake than microparticles (52). Also, on the nanoscale, smaller-size polyplexes are more able to enter cells and thereby increase the efficiency of transfection (53). Similarly, in 2004, Rejman *et al.* (54) showed with fluorescently labeled nanospheres (latex beads) that size itself can determine the pathway of entry. Ogris *et al.* (55) reported larger particles (1000 nm) exceeding the transfection efficiency achieved with smaller particles (40 nm) using DNA/transferrin–polyethylenimine complexes at physiological salt concentration. With a different DNA condensation system, they also reported that small toroid structures of DNA/transferrin–PLL complexes (80–100 nm) showed high transfection efficiency as their diameters are in the range of the coated pits involved in the endocytosis process (56).

We have previously reported (5–10) the importance of the substituents in the lipid moiety conjugated to the cationic polyamine to achieve improvements in DNA condensation efficiency for nonliposomal formulations where the lipid moiety must be considered in shape (volume) and substituent pattern, as well as the polyamine moiety and its pK_a values. The design and synthesis of novel cationic lipids based on the tetra-amine spermine, as nonliposomal formulations, where the lipid moiety is a long carbon chain, were largely instigated by Behr *et al.* (34) and Remy *et al.* (35) with their design and preparation of the highly efficient lipopolyamine DOGS (Transfectam®). A leading nonviral vector following from such structure–activity considerations is RPR120535 (57) where the di-C18 saturated alkyl chains are substituents pendant from a diamide at one end of the tetra-amine spermine, used as the cationic moiety to take advantage of DNA binding by polyamines (58). Unsaturated chains have

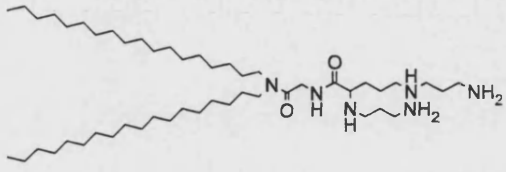
also been incorporated as the lipophilic moiety in lipopolyamine vectors.

We (11) and others (37) have used two covalently bound oleoyl chains in nonliposomal formulations for improved transfection efficiency by fusion with cellular membranes. In liposomal formulations, dioleoylphosphatidyl ethanolamine (DOPE) is often used as a helper lipid for its fusogenic ability as it has the characteristics of nonbilayer forming activity leading to destabilization of the lipid bilayer (59). Oleoyl and oleyl chains have also been bound as esters and ethers in liposomal formulations: DOSPER [1,3-dioleoyloxy-2-(6-carboxyspermine)] (60), DOSPA-DOPE (3:1 w/w, Lipofectamine™), and DOTMA-DOPE (1:1 w/w, Lipofectin®) (Fig. 7). Nonliposomal cationic-lipid delivery vectors combine both the characteristics of cationic and of helper lipids. So a factor that is important for both facilitating cell

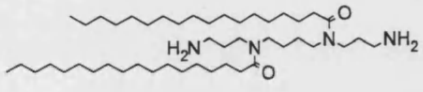
Spermine



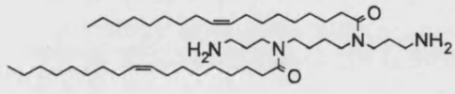
Transfectam® (DOGS)



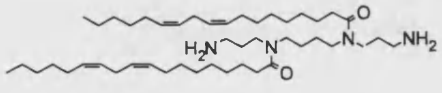
N^4,N^9-Distearoyl spermine



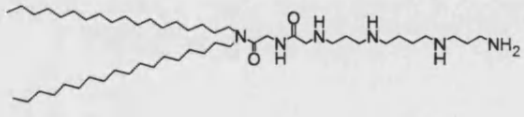
N^4,N^9-Dioleoyl spermine



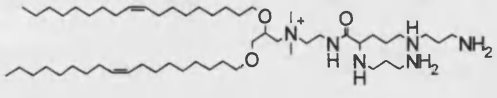
N^4,N^9-Dilinoleoyl spermine



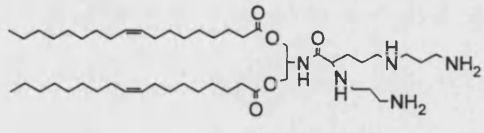
RPR120535



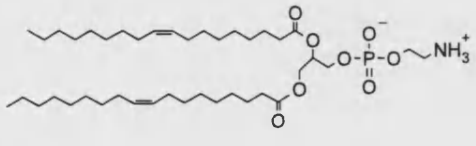
DOSPA



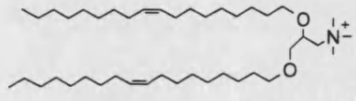
DOSPER



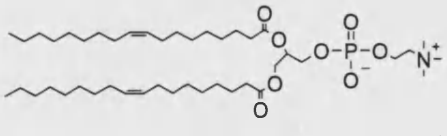
DOPE



DOTMA



DOPC



PE

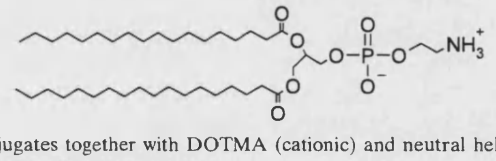


Fig. 7. Chemical structures of spermine and spermine conjugates together with DOTMA (cationic) and neutral helper lipids and gene delivery formulation excipients.

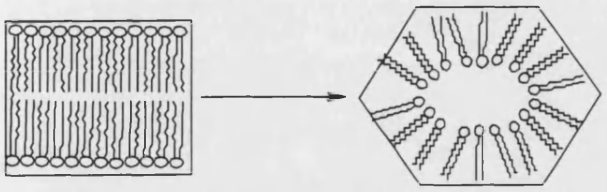


Fig. 8. Typical diagram of the change in lipid phase structure from the lamellar L_α phase to the inverted hexagonal H_{II} phase.

entry and the efficient intracellular release of free DNA (or lipoplexes) into the cytoplasm is the influence of the cationic lipid chain. The lipid moiety in our cationic lipids interacts with the phospholipid bilayer of the cell membrane and, either in crossing the membrane bilayer or in helping to weaken the endosomal bilayer, thereby aids the escape into the cytosol.

Cationic lipids in aqueous solutions exhibit different polymorphic phases. The two most important organizational forms are $L\alpha$ (lamellar organization with fluid hydrocarbon chains) and H_{II} (two-dimensional hexagonal state; Fig. 8) (61). Farhood *et al.* (59) have reported that the replacement of DOPE with a trimethylated structural analog dioleoylphosphatidyl choline (DOPC; Fig. 7), in cationic liposomal formulations, abolishes most of the transfection activity of the lipoplex, as DOPE exhibits a high tendency to form the inverted hexagonal (H_{II}) phase particularly at acidic pH.

Lipids that are cylindrical in solution shape prefer bilayer formations, whereas lipids with large head groups and small diameter hydrocarbon chains (inverted cones) form micellar structures (61). Lipids with small head groups and long hydrocarbon chains, e.g., (saturated) phosphatidyl ethanolamine (PE) and DOPE (Fig. 7), because of their solution cone shape, favor phase transition to an inverted hexagonal phase, as a result of steric factors (62). Also, increasing the degree of unsaturation of the acyl chain (especially in the *cis*-configuration) favors hexagonal phase formation ($L_\alpha \rightarrow H_{II}$ phase transition) and potentially improves the fusogenic characteristics of the lipid. The actual significance of maximizing such fusogenic characteristics in NVGT remains to be proven. Gaucheron *et al.* (63), in their detailed study on fluorinated lipospermines, speculated that the improved transfection with the unsaturated over the saturated counterparts could be a result of "their greater ability to promote membrane fusion with and destabilization of the endosomal membrane allowing optimal DNA release in the cytosol" and "related to the fluidification effect of the CC bonds." Vierling *et al.* (64) have also reported that unsaturated fluorinated lipids showed lower lamellar phase transition temperature than the corresponding fluorinated saturated lipids. The fusogenic characteristics of our synthetic nonliposomal lipopolyamines were taken under consideration to account for the observed difference in gene delivery. The two factors head group and chain length are held constant among our investigated compounds. The degree of unsaturation of the C18 fatty chain may be a key factor that explains the increase in the transfection efficiency of the lipoplex. This may be a result of the improved fusogenic characteristics of N^4,N^9-dilinoleoyl spermine with its (di-*cis*-configuration) dienoic fatty acyl residue, more than the monounsaturated N^4,N^9-dioleoyl spermine and the saturated N^4,N^9-distearoyl spermine. In conclusion, in this study, we have designed and synthesized novel gene carriers and characterized N^4,N^9-dilinoleoyl spermine as a promising DNA delivery formulation with its increased degree of unsaturation in both of the lipospermine fatty chains.

ACKNOWLEDGMENTS

We acknowledge the financial support of an Egyptian Government studentship to O.A.A.A. We are grateful to Prof. R. M. Tyrrell (University of Bath) for FEK4 and HtTA cell lines. We also thank Dr. J. M. H. van den Elsen (University of Bath) for generous help with the particle size measurements (funded under a BBSRC grant BBS/B/12121).

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BPC 2004 (POSTER)

Non-viral Formulation of DNA Using N^2, N^3-Dioleoyl Spermine

O. A. A. Ahmed and I. S. Blagbrough

Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.

prsisb@bath.ac.uk

As non-viral DNA-delivery vectors, cationic lipids and polymers have the ability to condense DNA into particles. These can be readily endocytosed by tissue-cultured cell lines and ultimately delivered to the nucleus. Our aims are the design and formulation of a novel non-viral vector capable of efficiently and safely delivering DNA to the nucleus in a variety of cell lines. The characteristics of lipospermines as cationic and lipophilic substances render these compounds promising carriers for DNA through the formation of lipoplexes (Blagbrough et al 2003). In this study, we synthesized and characterized a novel lipospermine in which the tetra-amine spermine (the cationic moiety) and dioleoyl chains (the lipophilic moiety) are linked by amide bonds at the secondary amino groups of spermine to form previously unreported N^2, N^3-dioleoyl spermine (LipoGen®). As among the prerequisites for delivery of DNA across intact cytoplasmic membranes are masking the negative charge of the phosphate backbone leading to DNA condensation, we studied the ability of N^2, N^3-dioleoyl spermine to condense calf thymus DNA and plasmid DNA (β-galactosidase, Promega) using an ethidium bromide (EthBr) (λ_{ex} = 260 nm, λ_{em} = 600 nm) fluorescence-quenching assay (Gershon et al 1993, Geall & Blagbrough 2000) in comparison with poly-L-lysine (PLL, average molecular weights 9.6 and 27 kDa) and polyethylenimine (PEI, average molecular weights 2 and 60 kDa) as model DNA condensing agents. To quantify the ability of these polyamines in DNA condensation, the binding constants of these polyamines with DNA was calculated. Also, particles of condensed DNA were detected using a UV light scattering assay at λ = 320 nm. The results indicate the ability of N^2, N^3-dioleoyl spermine to condense DNA by the significant decrease in (intercalated) EthBr fluorescence intensity and the increase in apparent absorption in light scattering experiments. The transfection results revealed high transfection efficiency in both an immortalized cancer cell line (HeLa) and in primary skin cells (FEK4) (in culture) using plasmid DNA encoding for enhanced green fluorescent protein (pEGFP) (Clontech) as the reporter macromolecule with its fluorescent imidazolidinone moiety analysed by FACS. The transfection efficiency and toxicity of N^2, N^3-dioleoyl spermine were studied in comparison with other commercially available liposomal cationic lipids Lipofectin® and Lipofectamine™. The DNA condensation and gene delivery using N^2, N^3-dioleoyl spermine was achieved at a small ammonium/phosphate (N/P) charge ratio (2.5 +/-) that minimises the toxic effects observed at higher N/P charge ratios. These results obtained with novel N^2, N^3-dioleoyl spermine show it to be efficient in both lipoplex formation and lipofection.

We acknowledge the financial support of the Egyptian Government (studentship to O.A.A.A.). We are grateful to Prof R. M. Tyrrell for the FEK4 cell line and to Dr C. Pourzand (University of Bath) for help in the cell biology studies and for useful discussions.

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BPC 2005 (POSTER)

Varying the Unsaturation in C18-Lipid Moieties of Spermine-Based Cationic Lipids for More Efficient Non-Viral Gene Delivery

Osama A. A. Ahmed, Charareh Pourzand and Ian S. Blagbrough

Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.

prsisb@bath.ac.uk

The use of an efficient vector for nucleic acid delivery is one of the determining factors for the successful application of gene therapy to difficult-to-treat diseases. Among non-viral delivery systems, non-liposomal cationic lipids are promising, non-toxic gene carriers. The synthesis by Behr et al (1989) of the lipopolyamine dioctadecylamidoglycylspermine (DOGS, Transfectam), as an efficient non-viral transfection agent, encouraged several laboratories to focus on the synthesis of novel cationic lipids based on the naturally occurring polyamine spermine. In an effort to improve DNA delivery, we investigated novel spermine-based cationic lipid formulations. Following on from our promising results obtained with N^4,N^9-dioleoyl spermine (Ahmed et al 2005), we have investigated the effect of the degree of unsaturation in the lipophilic moiety of our novel lipopolyamines on the overall transfection efficiency. The synthesis of saturated C18 (N^4,N^9-distearoyl spermine), and the doubly-unsaturated C18 (cis 9, 12) N^4,N^9-dilinoleoyl spermine followed that of the mono-unsaturated C18 (cis 9) N^4,N^9-dioleoyl spermine (Ahmed et al 2005). The binding affinities of these compounds for calf thymus DNA were determined using an ethidium bromide (EthBr) fluorescence-quenching assay (λ_{ex} = 260 nm, λ_{em} = 600 nm, Geall & Blagbrough 2000) utilising the ability of EthBr as a cationic dye that displays a marked increase in fluorescence on binding with DNA through the intercalation of the EthBr phenanthridinium moiety between adjacent base-pairs along DNA sequences. The increase in the apparent absorbance due to light scattering as a result of the formation of condensed DNA nanoparticles was also detected at λ = 320 nm. N^4,N^9-Dilinoleoyl spermine induced a marked decrease in intercalated EthBr fluorescence intensity from 100% (EthBr-DNA complex without cationic lipid) reduced to 10% at an ammonium/phosphate (N/P) charge ratio of 1.5 and a maximum (100%) relative apparent absorption at this same charge ratio. The transfection efficiencies of the synthesized lipopolyamines were studied in primary skin cells (FEK4 and FCP cells) and in an immortalized (HeLa derived HtTA) cancer cell line using plasmid DNA encoding for enhanced green fluorescent protein (pEGFP, Clontech) as the reporter macromolecule with its fluorescent imidazolidinone moiety analysed by Fluorescent Activated Cell Sorter (FACS). The results revealed improved transfection with N^4,N^9-dilinoleoyl spermine, typically 90% efficiency in primary cell lines, higher values than were obtained with the saturated N^4,N^9-distearoyl spermine, about 25-40% transfection efficiency in primary cell lines. The cytotoxicity of these compounds was studied in both primary skin and immortalised cancer cell lines using an MTT assay (about 70% viability at N/P 5.5). All these studies were compared with commercially available non-liposomal cationic lipid DOGS (Transfectam, Promega, typically 75% transfection and 75% cell viability at N/P 7.8). The results show that an increase in the number of double bonds in the long C18 fatty chain of the cationic lipid improves the ability of the non-viral vector to deliver the DNA payload to the cultured cells.

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BPC 2005 (POSTER)

N^4,N^9-Dimyristoyl Spermine is a Non-Viral Cationic Lipid Vector for Plasmid DNA Formulation

Osama A. A. Ahmed, Charareh Pourzand and Ian S. Blagbrough

Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.

prsisb@bath.ac.uk

The design and formulation of novel non-viral gene transfer (nano) particles have to overcome different barriers from the site of administration until they actively transport the DNA payload to the nucleus of target cells. Cationic lipids, as a major class of non-viral gene carriers, can be further classified as liposomal and non-liposomal non-viral DNA-delivery vectors. Lipopolyamines, especially those based upon the tetra-amine spermine, are promising non-viral gene delivery vectors, with significantly less toxicity and more controlled production than cationic polymers (Blagbrough et al 2003). These polyamine conjugates efficient condense DNA into nano-particles for cellular uptake by endocytosis, with ultimate DNA delivery to the nucleus in a variety of cell lines. In this study, we synthesized and formulated a novel non-liposomal lipospermine in which the tetra-amine spermine (the cationic moiety) and two C14 dimyristoyl fatty acid chains (the lipophilic moiety) are linked by amide bonds at the secondary amino groups of spermine to form N^4,N^9-dimyristoyl spermine. These amide linkers have the advantages of being both biodegradable and less toxic than the ether bonds in DOTMA (Tranchant et al 2004). Furthermore, the characteristics of this specific lipospermine with its cationic headgroup and two saturated long fatty-acid derived chains, render this conjugate a promising carrier for DNA-lipoplex formation. The binding affinity of this novel non-viral vector for calf thymus DNA (Sigma-Aldrich) was determined using an ethidium bromide fluorescence-quenching assay (λ_{ex} = 260 nm, λ_{em} = 600 nm, Geall & Blagbrough 2000). To quantify the ability of this polyamine conjugate in DNA condensation, the binding constant with DNA was calculated. Also, particles of condensed DNA were detected using a UV light scattering assay at λ = 320 nm. The results indicate the ability of N^4,N^9-dimyristoyl spermine efficiently to condense DNA by the significant decrease in (intercalated) EthBr fluorescence intensity and the increase in apparent absorption in light scattering experiments. The transfection results revealed high transfection efficiency in both an immortalized cancer cell line (HeLa derived HtTA cells) and in primary skin cell-lines (FEK4 and FCP cells in culture) using plasmid DNA encoding for enhanced green fluorescent protein (pEGFP) (Clontech) as the reporter macromolecule with its fluorescent imidazolidinone moiety analysed by Fluorescent Activated Cell Sorter (FACS). The viability of the cells was studied using an MTT assay. All the studies were investigated in comparison with the commercially available non-liposomal cationic lipid Transfectam (Promega). These results obtained with novel N^4,N^9-dimyristoyl spermine show it to be an efficient non-viral gene delivery vector for both lipoplex formation and lipofection.

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EUFEPS 2005 (POSTER)

Efficient non-viral gene delivery using unsaturated C18-lipid conjugates of spermine

Osama A. A. Ahmed, Charareh Pourzand, and Ian S. Blagbrough
Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.

prsisb@bath.ac.uk

The use of an efficient vector for nucleic acid delivery is one of the determining factors for the successful application of gene therapy to difficult-to-treat diseases. Among non-viral delivery systems, non-liposomal cationic lipids are promising, non-toxic gene carriers. The synthesis of the lipopolyamine dioctadecylamidoglycylspermine (DOGS, Transfectam) by Behr and co-workers (Behr et al 1989) as an efficient non-viral transfection agent, encouraged several laboratories to focus on the synthesis of novel cationic lipids based on the naturally occurring polyamine spermine. In an effort to improve DNA delivery, we are investigating novel spermine-based cationic lipid formulations. Following on from our promising results obtained with N^4,N^9-dioleoyl spermine (Ahmed et al 2005), we have investigated the effect of the degree of saturation in the lipophilic moiety of our novel lipopolyamines on the overall transfection efficiency. The synthesis of saturated C18 (N^4,N^9-distearoyl spermine), and the double-unsaturated C18 (cis 9, 12) N^4,N^9-dilinoleoyl spermine followed that of the mono-unsaturated C18 (cis 9) of N^4,N^9-dioleoyl spermine (Ahmed et al 2005). The binding affinities of these compounds for calf thymus DNA were determined using an ethidium bromide (EthBr) fluorescence-quenching assay (λ_{ex} = 260 nm, λ_{em} = 600 nm, Geall & Blagbrough 2000) utilising the ability of EthBr as a cationic dye that displays a marked increase in fluorescence on binding with DNA through the intercalation between the EthBr phenanthridinium-moiety and adjacent base-pairs along DNA sequences. The increase in the apparent absorbance due to light scattering as a result of the formation of condensed DNA nanoparticles was detected at λ = 320 nm. The transfection efficiencies of the synthesized lipopolyamines were studied in primary skin cells (FEK4 and FCP cells) and in an immortalized cancer cell line (HeLa derived HtTA cells, stably transfected with a tetracycline-controlled transactivator) using plasmid DNA encoding for enhanced green fluorescent protein (pEGFP) (Clontech) as the reporter macromolecule with its fluorescent imidazolidinone moiety analysed by Fluorescent Activated Cell Sorter (FACS). The cytotoxicity of these compounds was studied in both primary skin and immortalised cancer cell lines using an MTT assay. All the studies were compared with the commercially available non-liposomal cationic lipid Transfectam (DOGS, Promega). The results show that an increase in the number of double bonds in the long C18 fatty chain of the cationic lipid improves the ability of the non-viral vector to deliver the DNA payload to the cultured cells.

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EUFEPS 2005 (POSTER)

N^4,N^9-Dimyristoyl spermine is a non-viral cationic lipid vector for plasmid DNA formulation

Osama A. A. Ahmed, Charareh Pourzand, and Ian S. Blagbrough
Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.

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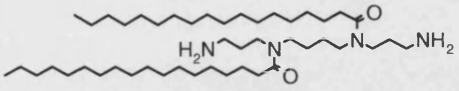
RSC Bioorg 2005 (ORAL)

Condensation and non-viral gene delivery to primary cells by designed lipopolyamines

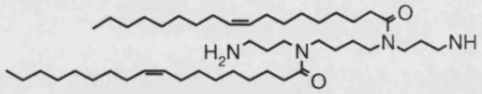
Osama A. A. Ahmed, Charareh Pourzand, and Ian S. Blagbrough* prsisb@bath.ac.uk

Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.

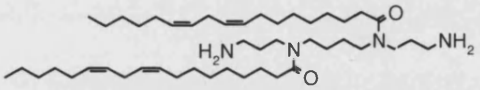
As viral gene therapy continues to suffer significant mammalian toxicity problems (1, 2), the possibility of reaching the goal of intracellular protein levels at therapeutic concentrations moves even more towards utilising non-viral gene therapy (NVGT). Among non-viral delivery systems, non-liposomal cationic lipids are promising, non-toxic gene carriers. We have designed novel spermine-based lipopolyamines and studied the effect of the degree of unsaturation in the lipophilic moiety on the overall transfection efficiency. The synthesis of saturated C18 (N^4,N^9-distearoyl spermine), doubly-unsaturated C18 (*cis*-9, *cis*-12) N^4,N^9-dilinoleoyl spermine (3), and mono-unsaturated C18 (*cis*-9) N^4,N^9-dioleoyl spermine (4) uses N^1,N^{12}-ditrifluoroacetyl spermine as a practical, selectively protected intermediate, amide synthesis, followed by NH_3/MeOH deprotection.



N^4,N^9-Distearoyl spermine



N^4,N^9-Dioleoyl spermine



N^4,N^9-Dilinoleoyl spermine

DNA condensation was studied using an ethidium bromide (EthBr) fluorescence-quenching assay (λ_{ex} = 260 nm, λ_{em} = 600 nm) (5). N^4,N^9-Dilinoleoyl spermine induced a marked decrease in intercalated EthBr fluorescence intensity from 100% (EthBr-DNA complex without cationic lipid) reduced to 10% at an ammonium/phosphate (N/P) charge ratio of 1.5. Light scattering by condensed DNA nanoparticles was detected at λ = 320 nm and a maximum (100%) relative apparent absorption (UV scattered light) at N/P charge ratio of about 1.5. The transfection efficiencies of the synthesized lipopolyamines were studied in primary skin cells (FEK4 and FCP cells) and in an immortalized (HeLa derived HtTA) cancer cell line using plasmid DNA encoding for enhanced green fluorescent protein (pEGFP) as the reporter macromolecule with its fluorescent imidazolidinone moiety analysed by Fluorescent Activated Cell Sorter (FACS). The results revealed high transfection with N^4,N^9-dilinoleoyl spermine, typically 85-90% efficiency in primary cell lines, higher values than were obtained with the saturated N^4,N^9-distearoyl spermine about 25-40%, N^4,N^9-dioleoyl spermine about 60-75% transfection efficiency. The cytotoxicity of these compounds was studied in both primary skin and immortalised cancer cell lines using an MTT assay (about 70% viability at N/P charge ratio 5.5). These results were compared with non-liposomal cationic lipid Transfectam, around 46-79% transfection, in the various cell lines, and 75% cell viability at N/P charge ratio 5.9. Naked (uncomplexed circular) pEGFP DNA typically gave 1-2% transfection of these cell lines. The results show that an increase in the number of double bonds in the long C18 fatty chain of the cationic lipid improves the ability of the non-viral vector to deliver the DNA payload to the cultured cells.

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5th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology 2006 (POSTER)

Varying the Unsaturation in C18-Lipid Moieties of Spermine-Based Cationic Lipids for More Efficient Non-Viral Gene Delivery

Osama A. A. Ahmed, Charareh Pourzand, and Ian S. Blagbrough
Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.
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Introduction

Among non-viral delivery systems, non-liposomal cationic lipids are promising, non-toxic gene carriers. The synthesis of dioctadecylamidoglycylspermine (DOGS, Transfectam) by Behr and co-workers [1] as an efficient non-viral transfection agent, encouraged several laboratories to focus on the synthesis of novel cationic lipids based on the naturally occurring polyamine spermine. The use of an efficient vector for nucleic acid delivery is one of the determining factors for the successful application of gene therapy to difficult-to-treat diseases. In an effort to improve DNA delivery, we are investigating novel spermine-based cationic lipid formulations. Following on from our promising results obtained with N^4,N^9-dioleoyl spermine [2], we have investigated the effect of the degree of unsaturation in the lipophilic moiety of our novel lipopolyamines on the overall transfection efficiency, achieved by the synthesis of saturated C18 (N^4,N^9-distearoyl spermine), doubly-unsaturated C18 (*cis*-9, 12) N^4,N^9-dilinoleoyl spermine, and mono-unsaturated C18 (*cis*-9) N^4,N^9-dioleoyl spermine [2].

Experimental methods

Synthesis of lipospermines

Spermine was used as the starting material for the synthetic process. The tetra-amine spermine was protected on both the primary amino functional groups with ethyl trifluoroacetate. Fatty chains were added to the secondary amino group using fatty acyl chloride. The tetra-amide was dissolved in methanol and the pH of the solution was increased by saturating with ammonia gas (deprotection of the primary amine groups). After silica gel column chromatography, all three synthesized lipopolyamines were homogeneous by thin-layer chromatography.

DNA condensation

DNA condensation was monitored using an ethidium bromide (EthBr) fluorescence quenching assay (λ_{ex} = 260 nm, λ_{em} = 600 nm [3]) utilising the ability of EthBr as a cationic dye that displays a marked increase in fluorescence on binding with DNA through the intercalation between the EthBr phenanthridinium-moiety and adjacent base-pairs along DNA sequences. Also, the increase in the apparent absorbance due to light scattering as a result of the formation of condensed DNA nanoparticles was detected at λ = 320 nm.

Lipoplex particle size and zeta potential measurements

The average particle size and ζ-potential measurements (at their optimum charge ratio of transfection) were determined using a Malvern Zetasizer (Nano S or ZS, Malvern Instruments, UK). All measurements were carried out on lipoplexes with 5 µg/ml plasmid DNA in HEPES buffer at pH 7.4 and 20 °C.

Transfection experiments

The transfection efficiencies of the synthesized lipopolyamines were studied in primary skin cells (FEK4 and FCP cells) and in an immortalized cancer cell line (HeLa derived HtTA cells, stably transfected with a tetracycline-controlled transactivator) using plasmid DNA encoding for enhanced green fluorescent protein (pEGFP) as the reporter macromolecule with its fluorescent imidazolidinone moiety (and analysis by FACS).

In vitro cytotoxicity

The cytotoxicity of these compounds was studied in both primary skin and immortalised cancer cell lines using an MTT assay.

Results and Discussion

DNA condensation

Three cationic lipid formulations (N^4,N^9-dilinoleoyl spermine, N^4,N^9-dioleoyl spermine, and Transfectam®) show similar DNA condensing ability. They are able to condense DNA completely (defined as more than 90% EthBr fluorescence quenching [4] at N/P charge ratio 2, while N^4,N^9-distearoyl spermine is only able to displace around 80% of EthBr at the same charge ratio.

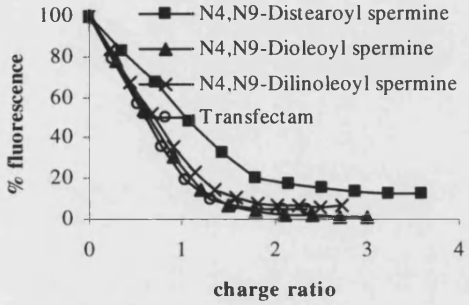

Figure 1. Plot of EthBr displacement assay of calf thymus DNA complexed with N^4,N^9-dilinoleoyl spermine, N^4,N^9-dioleoyl spermine, N^4,N^9-distearoyl spermine, and Transfectam®.

Lipoplex particle size and zeta potential measurements

Particle size characterization showed that the average particle size of N^4,N^9-dilinoleoyl spermine was considerably smaller (71 nm) than that of N^4,N^9-distearoyl spermine and N^4,N^9-dioleoyl spermine (366 nm). The surface charge, as determined by ζ-potential measurements, was +43 and +32 mV for N^4,N^9-dilinoleoyl spermine (at N/P charge ratio of 5.5) and N^4,N^9-dioleoyl spermine (at N/P charge ratio of 2.5) lipoplexes respectively.

Transfection experiments

The results indicate the improved transfection ability of N^4,N^9-dilinoleoyl spermine, greater than 85% in many of the studied primary cell lines (80% for FCP8 cells) and 99% in the case of HtTA cells compared to the saturated N^4,N^9-distearoyl spermine (18-32%), the mono-unsaturated N^4,N^9-dioleoyl spermine (62-75%), and Transfectam® (46-79%).

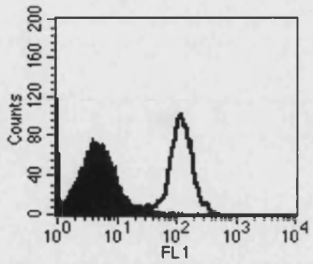

Figure 2. FACS analysis of FEK4 cells after 48 h transfection of pEGFP complexed with N^4,N^9-dilinoleoyl spermine: ■ untransduced cells, □ EGFP positive cells.

In vitro cytotoxicity

Cell viability results indicate that there is no significant difference in lipoplex toxicity in the case of HtTA and FCP8 primary skin cells for all four studied lipopolyamines. N^4,N^9-Dilinoleoyl spermine typically shows 70% cell viability in most of the studied cell lines, and such a value has recently been reported to be acceptable for a safe DNA delivery vector [5].

Conclusion

The results show that an increase in the number of double bonds in the long C18 fatty chain of the cationic lipid improves the ability of the non-viral vector to deliver the DNA payload to cultured cells.

Acknowledgements

We acknowledge the financial support of the Egyptian Government (studentship to O.A.A.A.). We are also grateful to Prof R. M. Tyrrell (University of Bath) for the FEK4 and HtTA cell lines.

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5th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology 2006 (POSTER)

N^4,N^9-Dimyristoyl Spermine is a Non-viral Cationic Lipid Vector for Plasmid DNA Formulation

Osama A. A. Ahmed, Charareh Pourzand, and Ian S. Blagbrough
Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.
prsisb@bath.ac.uk

Introduction

The formulation of novel non-viral gene transfer (nano) particles has to overcome different barriers from the site of administration until they actively transport the DNA payload to the nucleus of target cells. Cationic lipids, as a major class of non-viral gene carriers, can be further classified as liposomal and non-liposomal non-viral DNA-delivery vectors. Lipopolyamines, especially those based upon the tetra-amine spermine, are promising non-viral gene delivery vectors, with significantly less toxicity and more controlled production than cationic polymers [1, 2]. These polyamine conjugates efficient condense DNA into nano-particles for cellular uptake by endocytosis, with ultimate DNA delivery to the nucleus in a variety of cell lines. In this study, we synthesized and formulated a novel non-liposomal lipospermine in which the tetra-amine spermine (the cationic moiety) and two C14 dimyristoyl fatty acid chains (the lipophilic moiety) are linked by amide bonds at the secondary amino groups of spermine to form N^4,N^9-dimyristoyl spermine. These amide linkers have the advantages of being both biodegradable and less toxic than the ether bonds in DOTMA [3]. Furthermore, the molecular characteristics of this specific lipospermine with its cationic headgroup and two saturated long fatty-acid derived chains, render this conjugate a promising carrier for DNA-lipoplex formation.

Experimental methods

Synthesis of lipospermines

The tetra-amine spermine was protected on both the primary amine functional groups using ethyl trifluoroacetate. Dimyristoyl chains were added to the secondary amine groups using myristoyl chloride. Then, the tetra-amide was dissolved in methanol and the pH of the solution was increased by saturating with ammonia gas to remove the protecting groups, followed by purification to homogeneity using silica gel column chromatography.

Ethidium bromide and light scattering experiments

The binding affinity of this novel non-viral vector for calf thymus DNA (Sigma-Aldrich) was determined using an ethidium bromide fluorescence-quenching assay (λ_{ex} = 260 nm, λ_{em} = 600 nm [4]. To quantify the ability of this polyamine conjugate in DNA condensation, the binding constant with DNA was calculated. Also, particles of condensed DNA were detected using a UV light scattering assay at λ = 320 nm.

Lipoplex particle size and zeta potential measurements

The average particle size and ζ-potential measurements (at their optimum charge ratio of transfection) were determined using a Malvern Zetasizer (Nano S or ZS, Malvern Instruments, UK). All measurements were carried out on lipoplexes with 5 μg/ml plasmid DNA in HEPES buffer at pH 7.4 and 20 °C.

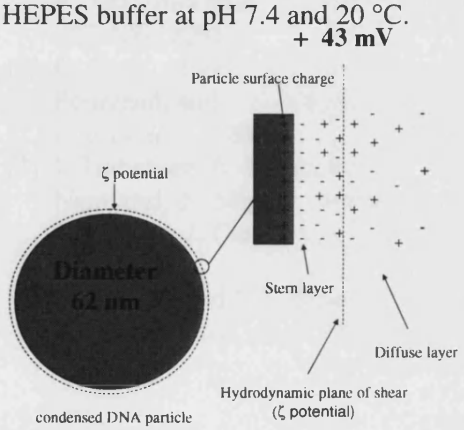

Figure 1. Diagram illustrating the particle size and ζ potential of the nanoparticles formed.

Transfection experiments and cytotoxicity

The transfection efficiencies of the synthesized lipopolyamines were studied in primary skin cells (FEK4 and FCP cells) and in an immortalized cancer cell line (HeLa derived HtTA cells, stably transfected with a tetracycline-controlled transactivator) using plasmid DNA encoding for enhanced green fluorescent protein (pEGFP) as the reporter macromolecule with its fluorescent imidazolidinone moiety analysed by FACS. The cytotoxicity of these compounds was studied in both primary skin and immortalised cancer cell lines using an MTT assay.

Results and Discussion

Ethidium bromide and light scattering experiments

The results indicate the ability of N^4,N^9-dimyristoyl spermine efficiently to condense DNA by the significant decrease in (intercalated) EthBr fluorescence intensity and the increase in apparent absorption in light scattering experiments.

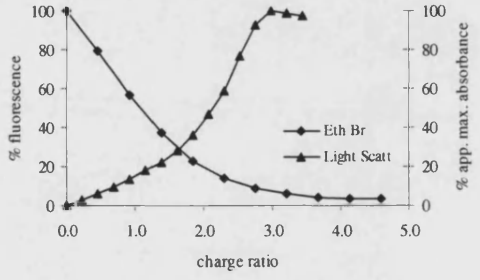

Figure 2. Comparison of ethidium bromide fluorescence quenching assay (λ_{excit} = 260 nm and λ_{emiss} = 600 nm with slit width 5 nm) and light scattering assay (% relative maximum apparent absorbance at λ = 320 nm) of N^4,N^9-dimyristoyl spermine complexed with pEGFP.

Lipoplex particle size and zeta potential measurements

N^4,N^9-Dimyristoyl spermine-pEGFP complex at N/P ratio (+/-) 15, shows an average particle size of 62 nm and has a ζ-potential of + 43 mV (at the optimum charge ratio of transfection).

Transfection experiments and cytotoxicity

The transfection results revealed high transfection efficiency in both an immortalized cancer cell line (HeLa derived HtTA cells) and in primary skin cell-lines (FEK4 and FCP cells in culture) in comparison with the commercially available non-liposomal cationic lipid Transfectam (Promega). Cell viabilities in the investigated cell lines were around 50%.

Conclusion

These results obtained with novel N^4,N^9-dimyristoyl spermine show it to be an efficient non-viral gene delivery vector for both lipoplex formation and lipofection.

Acknowledgements

We acknowledge the financial support of the Egyptian Government (studentship to O.A.A.A.). We are grateful to Prof R. M. Tyrrell (University of Bath) for the HtTA and FEK4 cell lines.

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BPC 2006 (POSTER)

Formulation and delivery of p-EGFP DNA condensed by a synthetic lipospermine

Osama A. A. Ahmed and Ian S. Blagbrough

Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.

prsisb@bath.ac.uk

Cationic lipids are non-viral DNA-delivery vectors. These vectors have the ability to condense DNA into particles which can be readily endocytosed in tissue-cultured cell lines. Ultimately, the DNA pay-load is delivered to the nucleus. The aim of this study is DNA formulation with a lipospermine non-viral vector capable of efficiently delivering the desired plasmid to the target nuclei in a variety of cell lines e.g. primary skin and cancer cells (Ahmed et al 2005 and 2006). We have synthesized and characterized a lipopolyamine in which the tetra-amine spermine, the cationic moiety, and two oleoyl chains as the lipophilic moiety are linked by amide bonds at the secondary amino groups of spermine to form N^4,N^9-dioleoyl spermine. Among the prerequisites for delivery of DNA across intact cytoplasmic membranes are masking the negative charge of the phosphate backbone leading to DNA condensation, we have therefore studied the ability of N^4,N^9-dioleoyl spermine to condense linear calf thymus DNA and circular plasmid DNA (β-galactosidase) using an ethidium bromide (EthBr, λ_{ex} = 260 nm, λ_{em} = 600 nm) fluorescence-quenching assay (Geall & Blagbrough 2000) and compared the results with those obtained using poly-L-lysine (PLL, average molecular weights 9.6 and 27 kDa) and polyethylenimine (PEI, average molecular weights 2 and 60 kDa) as model DNA condensing agents. Our results show that N^4,N^9-dioleoyl spermine is able to condense DNA with > 90% fluorescence quenching at ammonium/phosphate N/P charge ratio 2 more efficiently than PLL9.6 and the commercially available formulations Lipofectin® and Lipofectamine™. The DNA binding constants of these lipopolyamines were calculated, and particles of condensed DNA were detected using a UV light scattering assay at λ = 320 nm. N^4,N^9-Dioleoyl spermine is able to condense DNA, shown by the significant decrease in (intercalated) EthBr fluorescence intensity and the related increase in apparent absorption in the light scattering experiments. The transfection efficiency and toxicity of non-liposomal N^4,N^9-dioleoyl spermine were also studied in comparison with commercially available liposomal cationic lipid formulations Lipofectin® and Lipofectamine™. Transfection was performed using plasmid DNA encoding for enhanced green fluorescent protein (pEGFP) as the reporter macromolecule, with its fluorescent imidazolidinone moiety analysed by FACS. We found higher transfection efficiency in both primary skin cells (FEK4, 75%) and in an immortalized cancer cell line in culture (HtTA HeLa, 70%) than Lipofectin® that showed 18% and 58% transfection efficiency respectively. Our synthetic lipospermine showed comparable results with Lipofectamine™ (66% and 70%) in FEK4 and HtTA cells respectively. Using the cationic lipid N^4,N^9-dioleoyl spermine, both DNA condensation and gene delivery were achieved at a small N/P charge ratio (2.5 +/-) minimising the toxic effects seen at higher charge ratios (MTT assay). From our results, we conclude that N^4,N^9-dioleoyl spermine is efficient in both lipoplex formation and lipofection.

We acknowledge the financial support of the Egyptian Government (studentship to O.A.A.A.). We are grateful to Prof R. M. Tyrrell (University of Bath) for the HtTA and FEK4 cell lines and to Dr C. Pourzand (University of Bath) for help in the cell biology studies and for useful discussions.

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